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<b>(21) International Application Number:</b> PCT/GB92/01187 <b>(22) International Filing Date:</b> 1 July 1992 (01.07.92)  <b>(30) Priority data:</b> 9114259.6                      2 July 1991 (02.07.91)                      GB  <b>(71) Applicant (for all designated States except US):</b> IMPERIAL CHEMICAL INDUSTRIES PLC [GB/GB]; Imperial Chemical House, Millbank, London SW1P 3JF (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> BRIDGES, Ian, George [GB/GB]; 3 Ashes, Bramley Road, Silchester, Hamp- shire RG7 2LL (GB). BRIGHT, Simon, William, Jona- than [GB/GB]; 24 Pound Lane, Marlow, Buckingham- shire SL7 2AY (GB). GREENLAND, Andrew, James [GB/GB]; The Cabin, Raymill Road East, Maidenhead, Berkshire SL6 85X (GB). HOLT, David, Charles [GB/ GB]; 22 Easthampstead Road, Wokingham, Berkshire RG11 2EG (GB). JEPSON, Ian [GB/GB]; 41 Grays Road, Slough, Berkshire SL1 3QG (GB). SCHUCH, Wolfgang, Walter [DE/GB]; 14 Greenfinch Close, Heathlake Park, Crowthorne, Berkshire RG11 6TZ (GB).		<b>(74) Agent:</b> HUSKISSON, Frank, Mackie; Imperial Chemical Industries plc, Group Patents Services Department, Shire Park, P.O. Box 6, Bessemer Road, Welwyn Garden City, Herts AL7 1HD (GB).  <b>(81) Designated States:</b> AU, BB, BG, BR, CA, CS, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, PL, RO, RU, SD, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PLANT-DERIVED ENZYME AND DNA SEQUENCES, AND USES THEREOF  <b>(57) Abstract</b>  The chemically-inducible 27 kD subunit of the enzyme glutathione-S-transferase, isoform II (GST-II-27) and sequences encoding it are provided. In particular, a genomic DNA sequence encoding the gene promoter for the GST-II-27 subunit is provided. When linked to an exogenous gene and introduced into a plant by transformation, the GST-II-27 promoter provides a means for the external regulation of expression of that exogenous gene. Transformation with DNA encoding glutathione-S-transferase polypeptides produces herbicide resistance transgenic plants.		

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PLANT-DERIVED ENZYME AND DNA SEQUENCES, AND USES THEREOF

This invention relates to a glutathione-S-transferase enzyme and DNA sequences coding for it.

Glutathione-S-transferases (GST) are a family of enzymes which catalyse the conjugation of glutathione, via a sulphhydryl group, to a large range of hydrophobic, electrophilic compounds. The conjugation can result in detoxification of these compounds and may result in their removal from tissue.

GST enzymes have been identified in a range of crop plants including maize, wheat, sorghum and peas. GST's comprise from 1 to 2% of the total soluble protein in etiolated maize seedlings.

Three isoforms of GST have been identified: GST-I, GST-II and GST-III. The major isoform in maize tissue, GST-I, is constitutively expressed and is capable of conjugating glutathione with pre-emergent herbicides such as alachlor.

Treatment of maize tissues with chemical safeners (for example, N,N-diallyl-2,2-dichloroacetamide) raises the activity of GST-I which participates in the detoxification of the pre-emergent herbicides.

International Patent Application WO 90/08826 describes GST-II (isoform II).

Both GST-I and GST-II proteins have a native molecular weight of approximately 50 kD. As in mammals, maize GST's are dimeric; GST-I has apparently identical polypeptide subunits of 29 kD (GST-I-29), whereas GST-II is a heterodimer of a 29 kD subunit identical to that found in GST-I (GST-I-29) and a novel 27 kD subunit (GST-II-27). GST-II is detected at a very low basal level in the absence of safener, but its expression is enhanced dramatically by safener treatment. Like GST-I,

GST-II confers resistance to certain herbicides. GST-II is known to detoxify chloroacetanilide and thiocarbamate herbicides such as alachlor (Mozer et al, 1983, Biochemistry, 22:1068-1072).

5           A cDNA and a gene corresponding to the 29 kD subunit of GST-I have been cloned previously and sequenced (Wiegand et al, 1986, Plant Mol Biol, 7:235-243). In addition, a cDNA corresponding to a 26 kD subunit of a third, minor component of GST  
10 activity in maize seedlings (GST-III-26) has been previously cloned and sequenced (Moore et al, 1986, Nucleic Acid Research, 18:7227-7235). GST-III is a homodimer of these 26 kD subunits. Like GST-I and unlike GST-II, GST-III is constitutively expressed.  
15 It is known to detoxify herbicides such as atrazine.

          According to the present invention, we provide a genomic DNA sequence encoding the gene promoter for the 27 kD subunit of the glutathione-S-  
20 transferase, isoform II, enzyme (GST-II-27), containing the nucleotide sequence shown in Figure 8 herewith and variants of the said sequence as permitted by the degeneracy of the genetic code.

          The genomic DNA was deposited on 14 June 1991  
25 in the National Collections of Industrial and Marine Bacteria (NCIMB), 23 St Machar Drive, Aberdeen, AB2 1RY, Scotland, UK, as plasmid pGIE7 contained within Escherichia coli, strain XLI-Blue with the accession number NCIMB 40426.

30           The invention also provides a GST-II-27 enzyme subunit having the amino acid sequence shown in Figure 4 herewith.

          The invention further provides a cDNA sequence encoding this GST-II-27 subunit, having the

nucleotide sequence shown in Figure 2 herewith and variants of the said sequence as permitted by the degeneracy of the genetic code.

The cDNA was deposited on 19 April 1991 in the National Collections of Industrial and Marine Bacteria (NCIMB), 23 St Machar Drive, Aberdeen, AB2 1RY, Scotland, UK, as plasmid pIJ21 contained within Escherichia coli, strain XLI-Blue with the accession number NCIMB 40413.

International Patent Application WO 90/08826 describes a chemically inducible gene promoter sequence isolated from a 27 kD subunit of the maize GST-II gene (GST-II-27). The said International application is incorporated herein by reference.

In the present application we describe the cloning and sequencing of a complete cDNA corresponding to the 27 kD subunit. This sequence is inducible and specifically recognised by a GST-II-27 specific antiserum. It is partially homologous with other maize GSTs.

By virtue of the present invention, the cDNA for GST-II-27 has been utilised as a gene probe for the isolation of a corresponding genomic sequence which includes the promoter region.

The invention further provides a chemically switchable gene construct which includes the GST-II-27 gene promoter operatively linked to a foreign gene or a series of foreign genes whereby expression of said foreign gene or said series of genes may be controlled by application of an effective exogenous inducer. The invention also provides plants transformed with said gene construct.

The GST-II-27 gene has been shown previously

(International Application Number WO 90/08826) to be induced by certain chemical compounds, known as "herbicide safeners", which can be applied, as a spray, for example, to growing plants. Induction may be achieved by application of any suitable chemical including known safeners and other agrochemicals, chemical analogues and other potential inducers. Such chemicals may include N,N-diallyl-2,2-dichloroacetamide (common name: dichloramid); 2,2,5-trimethyl-3-(dichloroacetyl)-1,3-oxazolidine; benzyl-2-chloro-4-(trifluoromethyl)-5-thiazole-carboxylate (common name: flurazole); naphthalene-1,8-dicarboxylic anhydride; 2-dichloromethyl-2-methyl-1,3-dioxolane; 1-(dichloroacetyl)-hexahydro-3,3,8a-trimethyl-pyrrole (1,2-a)-pyrimidin-6(2H)-one; 1,3-dioxolan-2-ylmethoxyimono(phenyl)benzene acetonitrile; 4,6-dichloro-2-phenyl-pyrimidine; 2,2-dichloro-[N-allyl-N(1,3-dioxalano-2-methyl)] acetamide; 1-(cyanomethoxyimino)benzacetone nitrile; 4'-chloro-2,2,2-trifluoroacetophenone-O-1,3-dioxolan-2-yl methyloxime; 2,2-dichloro-1-(3,4-dihydro-3-methyl-2H-1,4-benzoxazin-4-yl) ethanone; 3-dichloroacetyl-2,2-dimethyloxazolidine; 4-methoxy-3,3-dimethylbenzophenone; 1-cyclohexyl-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pent-1-en-3-ol; 2,2-dichloro-N-(3-methyl-4-thiazolin-2-ylidene) acetamide; O,O-diethyl-O-phenyl phosphorothioate; 2,2-spirocyclohexyl-N-dichloroacetyl oxazolidine; N-benzyl-N-ethyl-dichloroacetamide; 3-chloroacetyl-4,4-cyclohexane-spiro-2,2-dimethyl-1,3-oxazolidine; spirooxazolidine acetamide; oxabetrinil; cyometrinil; fenclorim;

methoxyphenone.

The GST-II-27 promoter, then, when linked to an exogenous or foreign gene and introduced into a plant by transformation, provides a means for the external regulation of expression of that foreign gene. The foreign gene may be any gene other than the wild type GST-II-27 gene. The said International Patent Application WO 90/08826 provides details of the manner of use of such an externally regulatable promoter and International Patent Application Number WO 90/08830 describes in detail the use of such promoter as a component of a gene cascade leading to regulation of male sterility in plants and the uses of such plants in the production of hybrid crops. It is shown here that the inducible GST-II-27 promoter is functional in both monocotyledons and dicotyledons. It can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, cotton; cereals such as wheat, barley, rice, maize, sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas and melons; and vegetables such as carrot, lettuce, cabbage and onion. The GST-II-27 promoter is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

In general outline, the procedures which were used in isolating the enzyme, the cDNA and the genomic DNA of this invention were as follows:

Total RNA was extracted from safener-induced maize tissue at the peak of GST activity, and used to construct a cDNA library which contained GST

transcripts.

The GST-II-27 protein was extracted from the same type of tissue, purified and used to raise a sheep antiserum (characterised by western and dot blot analysis).

By immuno-screening the cDNA library with this antiserum (using an isotopic detection system), the new GST sequence was isolated. Plaque purification allowed in vivo excision and sequencing of the plasmid DNA containing this GST. Northern analysis showed that it was a safener-inducible clone.

GST-II-27 genomic clones were isolated by screening a maize genomic library with a carefully designed probe prepared from the above cDNA. By mapping these clones, a fragment containing the promoter region was isolated and sequenced.

The promoter sequence was used to construct plant gene expression cassettes. These cassettes were introduced into both monocotyledons and dicotyledons and shown to control gene expression in an inducible manner.

The invention further provides a method to produce a herbicide resistant transgenic plant which comprises the incorporation of DNA encoding GST polypeptides into the plant such that a glutathione-S-transferase enzyme is expressed. The invention also provides herbicide resistant plants produced by said method, and their progeny.

The purpose in providing crop plants which resist the action of a herbicide is to facilitate the control of weeds growing between the plants by the overall application of an effective concentration of a herbicide which would destroy the crop plant in its normal, that is herbicide sensitive, state. Such resistant plants are also



useful for use in a locus of any short term  
carry-over of herbicide from a previous  
application. In this context, a resistant plant is  
defined as one which displays enhanced tolerance to  
5 a herbicide when compared to a standard plant.  
Resistance may vary from a slight increase in  
tolerance to the effects of the herbicide to total  
resistance where the plant is unaffected by the  
presence of herbicide.

10 Herbicidal tolerant plants containing a  
glutathione-S-transferase gene have been described  
in Australian patent Application 73146/87 (Ciba  
Geigy) published on 26 November 1987 (related to US  
patent number 5073677 published on 17 December  
15 1991). The patent application describes the use of  
mammalian GST sequences, specifically a rat  
sequence. The genetic sequence coding for a rat  
GST is disclosed and used to construct plant  
transformation vectors for tobacco; some  
20 transformants showed atrazine resistance. The  
patent application does not describe GST sequences  
derived from plants; in particular, it does not  
disclose the GST-II-27 sequence.

DNA encoding GST polypeptide subunits may be  
25 incorporated into a plant transformation vector  
under the control of a suitable promoter  
(constitutive or inducible). A plant expressing  
GST-I 29 kD polypeptide subunits will show GST-I  
enzyme activity. A plant co-expressing GST-I 29 kD  
30 subunits and GST-II 27 kD subunits will show GST-II  
enzyme activity, and some GST-I activity. A plant  
expressing GST-III 26 kD subunits will show GST-III  
enzyme activity. A plant co-expressing GST-I 29 kD  
and GST-III 26 kD subunits will show GST-I and  
35 GST-III activity. A plant co-expressing GST-I 29

kD, GST-II 27 kD and GST-II 26 kD polypeptide will show GST-I, GST-II and GST-III activity. All such plants will be capable of conjugating glutathione with certain pre-emergent herbicides: they will thus be resistant to these herbicides. GST activity is known to be effective against a range of chemical herbicides, including the chloroacetanilides and the thiocarbamates, and may be active against other compounds which show herbicidal properties. The actual spectrum of herbicide resistance displayed by the transgenic plant will depend on which GST isoform is active (GST-I, GST-II or GST-III) or on the relative activity of the various GST isoforms present in the plant. For example, GST-II is active against alachlor and acetochlor; GST-III is active against atrazine. Thus the method of producing herbicide resistant transgenic plants may be used not only to confer resistance on previously susceptible plants or to increase existing resistance, but also to broaden the range of herbicides to which the plant is resistant. So the ability to introduce multiple resistances by expressing the various GST isoforms within the transgenic plants is advantageous for agricultural purposes. Specific resistance to a particular herbicide may be achieved by incorporation of the specific GST isoform (or combination of isoforms) which is most effective against this herbicide. In particular, the presence of GST-II activity in the plant may provide resistance to certain herbicides which cannot be detoxified by the GST-I or GST-III enzymes. Thus the ability to produce plants in which herbicide resistance is conferred by GST-II activity (via transformation with the GST-II-27

sequence) will be advantageous.

Plants may be transformed with constructs containing sequences which encode GST subunits according to a variety of known methods

5 (Agrobacterium Ti plasmids, electroporation, microinjection, microprojectile gun, etc). The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the

10 genome. Both transformed monocot and dicot plants may be obtained in this way, although the latter are usually more easy to regenerate. Examples of genetically modified plants which may be produced include field crops such as canola, sunflower,

15 tobacco, sugarbeet, cotton and cereals such as wheat, barley, rice, sorghum and also maize.

Some plant species do not naturally contain GST enzymes which can detoxify herbicides. Incorporating DNA encoding GST polypeptides into

20 such species will thus confer new or additional resistance to certain herbicides. Although GSTs are naturally expressed in other species, notably maize, production of transgenic plants containing enhanced levels of GSTs may increase the crop's

25 level of herbicide resistance. In addition, all the GST isoforms are not normally present in all maize lines. Production of transgenic maize containing one or more additional GST isoforms will confer resistance to a wider range of herbicides.

30 A further advantage comes from placing the GST-II-27 sequence under control of a constitutive promoter, as this will mean that the GST-II enzyme is constitutively expressed within the transgenic maize plant. This avoids the need to apply a

35 chemical safener to the maize seed or plant.

Normally, GST-II enzyme activity is inducible. It is current practice to apply a safener (such as dichloramid) as a seed coating to induce GST-II enzyme activity in the emerging plant, thus conferring herbicide resistance.

Amino acid sequencing has shown that the 29 kD subunit of GST-I is identical to the 29 kD subunit of GST-II. Thus a combination of the GST-I-29 subunit and the GST-II-27 subunit will give an active GST-II enzyme. Co-expression of the cDNA encoding GST-I-29 (isolated by Wiegand et al, 1986, Plant Mol Biol, 7:235-243) and the cDNA encoding GST-II-27 (as shown in Figure 2) within a transgenic plant will give a plant with GST-II activity.

DNA encoding the GST-I 29 kD subunit or the GST-II 27 kD subunit may be incorporated into a vector under the control of a suitable promoter such as the 35S CaMV promoter. Plants may be transformed with vectors containing either the GST-I-29 or the GST-II-27 expression cassette. Transformants expressing the respective GST-II subunits (29 kD or 27 kD) may be crossed to produce progeny expressing both GST-I-29 and GST-II-27, resulting in a herbicide resistant phenotype. In a modification of this method, each plant may be co-transformed with vectors containing the GST-I-29 expression cassette and with vectors containing the GST-II-27 expression cassette. Alternatively, DNA encoding the GST-I 29 kD subunit and the GST-II 27 kD subunit may be included in a single plant transformation vector under the control of a single promoter. Transformants expressing both GST-II subunits (29 kD and 27 kD) will show a herbicide resistant phenotype; transformants expressing only

one of the respective GST-II subunits (29 kD or 27 kD) may be crossed to produce progeny expressing both subunits.

5 The above methods can be adapted to produce herbicide resistant plants expressing the GST-I enzyme (transformation with DNA encoding GST-I-29), the GST-II enzyme (transformation with DNA encoding GST-III-26, as isolated by Moore et al, 1986, Nucleic Acid Research, 18:7227-7235), or some  
10 selection of GST-I/GST-II/GST-III activity.

Preferably, DNA encoding the GST subunits is introduced into the plant under control of a constitutive promoter (such as the 35S CaMV promoter). This avoids any need for external  
15 induction of GST expression: the plant is permanently herbicide resistant. DNA encoding the GST subunits may also be included in a plant transformation vector under the control of an inducible promoter, to give inducible herbicide  
20 resistance in the transgenic plants. Such a promoter includes the chemically-inducible GST-II-27 promoter as shown in Figure 8.

Resistance may be switched on by application of a suitable inducer (such as a chemical safener). In  
25 certain circumstances, the ability to express or to increase herbicide resistance only when required may be advantageous. For example, during rotation of crops, individuals of the first crop species may grow the following year in the field to be  
30 cultivated with a second crop species. A herbicide may be used to destroy these un-induced and still susceptible "volunteer" plants. Induction of GST expression only when herbicide resistance is  
35 herbicide) may also be metabolically more efficient

in some circumstances as the plant is producing GST polypeptides only when required.

The invention will now be described by way of illustration in the following description, which gives details of the isolation, expression studies and sequencing of GST-II-27, the construction of inducible expression cassettes and demonstration of their functioning in transgenic plants, plus the production of herbicide resistant plants.

The drawings which accompany this application are as follows:

Figure 1 is a time course graph showing GST activity in induced and uninduced maize root tissue.

Figure 2 shows the nucleotide sequence of cDNA encoding GST-II-27.

Figure 3 shows the northern analysis of induced and uninduced RNA samples.

Figure 4 shows the amino acid sequence of GST-II-27 compared to the amino acid sequences of GST-I-29 and GST-III-26.

Figure 5 shows primer extension mapping of the genomic clones.

Figure 6 represents the 5' end of the GST-II-27 gene.

Figure 7 shows the strategy used to sequence 5.4 Kb of the GST-II-27 gene and promoter.

Figure 8 shows the nucleotide sequence of the GST-II-27 promoter.

Figure 9 gives an overview of GST GUS vector construction.

Figure 10 shows the maize transient transformation vector, pPUG.

Figure 11 shows the tobacco stable transformation vector, pGSTTAK.

Figure 12 shows the maize stable transformation vector, pZM/RMS-3.

5        Figure 13 shows results of the protoplast transient expression assays.

Figure 14 shows results of the tobacco stable transformation experiments.

10       Figure 15 shows results of the maize stable transformation experiments.

Figure 16 shows the tissue localisation of the GST II 27 kD subunit in untreated and safener treated maize.

15       Figure 17 shows GST II concentration following safener treatment of maize in field trials.

Figure 18 shows GST II concentration in variously-sized uninduced maize tassels compared to induced tissue.

20       Figure 19 illustrates preparation of the vectors containing the CaMV35S-GST-I-29 or CaMV35S-GST-II-27 cassette.

#### SAFENER TREATMENT OF CORN TISSUE

25       For treatment of young maize seedlings, seeds were germinated on moist filter paper. After germination and growth (up to one week) the safener N,N-diallyl-2,2- dichloroacetamide (hereinafter referred to as R-25788) was added to the water in the filter paper and the seedlings grown for a  
30       further time period before harvesting of tissue.

#### ENZYME ASSAY

Enzyme activity was measured spectrophotometrically at 340 nm using 1-chloro-

2,4-dinitrobenzene (CDNB) as a substrate. The reaction buffer contained 0.1M EDTA, 0.001M CDNB and 0.0025M glutathione.

5            PREPARATION OF EXTRACTS & ENZYME PURIFICATION

Tissue was homogenised in 0.05M Tris.HCl, pH 7.8; 0.001M EDTA; 0.001M DTT; and 7.5% polyvinylpyrrolidone in a pestle and mortar, at 4°C, and centrifuged at 30,000g to obtain a crude  
10        extract.

Separation of the GST isoforms from the crude extract was achieved as follows: the crude extract was applied to a DEAE Sepharose column and washed with 0.01M Tris.HCl, pH 7.8; 0.001M EDTA; and  
15        0.001M DTT. The bound GST was eluted with 0.3M potassium chloride. Fractions containing GST activity were combined and desalted using PD10 gel filtration columns. Separation of the GST I and GST II isoforms was achieved by FPLC on a mono-Q  
20        column and a zero to 0.4M potassium chloride concentration gradient.

Pure samples of GST I and GST II were obtained by applying desalted fractions of GST I and GST II from the FPLC to a glutathione-S-sepharose affinity  
25        column equilibrated with 0.05M phosphate buffer at pH 7.3. After washing with buffer, bound GST was eluted with 0.005M glutathione.

SDS-PAGE (17.5%, 30:0.174 acrylamide: bisacrylamide) of GST I or GST II was achieved by  
30        concentrating pure GST samples using Amicon Centricon 10 Microconcentrations (Trade Mark), denaturing samples in mercaptoethanol containing Laemmli buffer, and staining the gels with Coomassie Blue.



### INDUCIBLE EXPRESSION OF GST

A time course experiment was carried out to examine the expression of GST's after safener treatment. A 30 ppm solution of R-25788 was applied to three-day old seedling roots and tissue harvested after various time intervals following safener treatment. Samples were tested for GST activity using the enzyme assay described above. The results of this experiment are presented graphically in Figure 1. This shows there was an approximate 2.5 fold increase in total GST activity after 48 hours incubation.

### CONSTRUCTION OF cDNA LIBRARIES

The time course experiments revealed a peak of GST expression at 48 hours after treatment with safener. Therefore, two cDNA libraries were constructed from total RNA extracted from root tissue at 24 and 48 hours after safener treatment.

To ensure that the induction procedure had been successful, a one gram sample of 24 hour induced tissue was taken and assayed for GST II. This experiment revealed that the tissue used to construct the cDNA library had indeed been successfully induced as GST II accounted for about 25% of the total GST activity.

Double-stranded cDNA was prepared from oligo dT-cellulose-purified RNA by a method employing RNase and E coli DNA polymerase I in the synthesis of the second strand, without prior purification of single-stranded cDNA (Gubler and Hoffman, 1983). Lambda ZAP II was chosen as the cloning vector.

GENERATION OF ANTIBODIES TO THE GST-II-27ENZYME

Sufficient protein to enable the immunisation of sheep was obtained by pooling the enzyme subunit isolated as described above from a number of separate experiments. The 27 kD GST II polypeptide was subsequently purified to apparent homogeneity by electroelution from polyacrylamide gel slices. Antisera were prepared against the 27 kD polypeptide. The immunisation of sheep was carried out essentially according to Stewart and Rowe (1975, J Immunological Methods, 8:37-45).

Western blotting experiments showed that a specific recognition of the GST-II 27 kD subunit was obtained using an antiserum. Furthermore, no cross-reactivity with the GST-II 29 kD subunit was obtained. The affinity and specificity of the primary antiserum was tested by immuno dot blotting and western blotting, showing no cross reactivity with other polypeptides in crude extracts.

A variety of control experiments were carried out to optimise the primary and secondary antibody concentrations, to test various enzyme substrates, and to validate fusion protein induction conditions. Conditions which maximised the signal to background noise ratio were identified, and allowed detection of 1 ng GST-II 27 kD subunit.

IMMUNO-SCREENING OF THE cDNA LIBRARIES

In order to identify a cDNA clone encoding maize GST-II-27, bacteriophage from the cDNA library were screened.

Immuno-screening using a  $^{125}\text{I}$  protein G detection system allowed detection of 0.1 ng of

denatured GST-II-27.

1x10<sup>6</sup> recombinants from the safener induced seedling library were screened at high plating densities (3x10<sup>4</sup> per plate). 30 positive plaques were detected, picked and rescreened using overnight incubation with IPTG soaked filters. Six strongly positive candidates remained after 3 rounds of plaque purification.

#### 10            FURTHER PLAQUE PURIFICATION

A fourth round of plaque purification was carried out using immuno-screening and DNA hybridisation (with the cDNA probe isolated by PCR from the third screen). Six clones came through plaque purification with both detection methods. Furthermore, the DNA probe cross-hybridised at equal intensities with all 6 positive clones, indicating they probably represented the same sequence.

20

#### ISOLATION OF PLASMID DNA

The in vivo excision protocol (Stratagene) was carried out to liberate Bluescript phagemids. Plasmid DNA prepared from four different lysates was designated pIJ13, pIJ15, pIJ17 and pIJ21 respectively.

25

Plasmid pIJ21 was deposited in the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, with the accession number NCIMB 40413.

30

#### CHARACTERISATION OF LIBRARY POSITIVES CLONES

When digested with EcoRI all clones liberated a single insert of between 900-950 base pairs,

which is the predicted insert size to encode a 27 kD protein. When the gel was blotted and hybridised with pIJ17 all inserts hybridised with equal intensities.

5           The 950 base pair insert from one of the clones was hybridised to a Southern blot of GST-I-29 and GST-III-26 PCR products. The experiments showed no hybridisation to GST-III-26 and a very weak hybridisation to GST-I-29 (1µg  
10 target DNA with overnight exposure). When the Southern was stripped and rehybridised with a GST-I-29 specific probe, only a 15 minute exposure was required.

15           The 950 base pair insert (the putative GST-II-27 PCR product) and the GST-I-29 specific oligo were also used to probe library filters of  $1 \times 10^6$  recombinants. Different hybridisation patterns were obtained for each probe.

20           These studies indicate the positive clone from the library was not GST-I-29 or GST-III-26, but did share some weak homology with GST-I-29.

#### SEQUENCE ANALYSIS OF cDNA

25           Between 200-300 bases of the 3' and 5' ends of each clone was sequenced using SK and KS primers. These data showed the four clones (pIJ13, pIJ15, pIJ17 and pIJ21) to be identical, except for the position of the poly A tail. As with GST-I-29 three possible poly A addition sites were  
30 identified.

          The longest clone pIJ17KS was fully sequenced: the cDNA sequence (954 base pairs) is shown in Figure 2. The sequence of pIJ17KS shows homology in the central region with maize GST-I-29 and

GST-III-26, which provided strong evidence that this clone represents a maize GST. However, the region of high homology between GST-I-29 and GST-III-26 at the 5' end is dissimilar to the 5' region of pIJ17KS, showing that the latter codes for a different protein.

Analysis of the coding region of GST-II-27 shows it to be a G-C region and to contain several repeats in the 5' region.

#### EVIDENCE THAT pIJ17KS ENCODES AN INDUCIBLE GST

Expression studies carried out by northern analysis strongly suggested that pIJ17KS corresponds to a GST-II-27 sequence. pIJ17KS strongly hybridised to a 0.95 kB transcript in induced RNA (I) isolated from a range of treated tissues including roots (R), tassel (T), silks (S) and leaf (L). With uninduced RNA (U), there was a poor hybridisation signal or in some cases no signal, as shown in Figure 3. Control hybridisation with a constitutive probe indicated equal loadings of RNA for the induced and uninduced samples. This indicates that pIJ17KS indeed represents a safener inducible clone. Densitometry analysis shows a 100 fold induction in the 0.95 kB transcript in certain tissues such as leaf after safener treatment.

The presence of a signal in uninduced RNA supports previous western data which detected a low basal expression of GST-II 27 kD subunit in the absence of safener treatment.

AMINO ACID SEQUENCE OF GST-II-27

Pure samples of GST-II were obtained by Sulphobromophthalein Glutathione-S-Agarose affinity chromatography. To separate the 27 kD and 29 kD subunits, the GST-II was injected onto a C8 Reverse Phase HPLC column (SGE 100mm x 2.1mm , 300 Å pore). The subunits were eluted with a gradient of acetonitrile in 0.1% Trifluoroacetic Acid. SDS PAGE (17.5%T ,0.6%C ) identified the UV absorbing peaks that corresponded to the 27 kD and 29 kD subunits.

The total amino acid composition of each subunit was determined using an Applied Biosystems 420AH Derivatizer Automated Amino Acid Analyser. This showed that there was close agreement between the measured composition and that predicted from the nucleic acid sequence for both the 29 kD and 27 kD subunits.

N-terminal sequence analysis was performed on each subunit by Edman Degradation using an Applied Biosystems 477 Pulsed Liquid Phase Automated Amino acid Sequence Analyser. This showed that the N-terminal sequence of the 29 kD subunit is identical to the 29 kD subunit of GST-I. The N-terminus of the GST-II 27 kD subunit was blocked to Edman Degradation.

Reverse phase HPLC separated GST-II-27 was freeze dried in a Univap freeze concentrator. Each subunit was reduced using 6M Guanidine-HCL and 45mM Dithiothreitol at 50°C for 15 minutes and then alkylated with 8mM Iodoacetamide at 20°C for 15 minutes. The protein was diluted to produce a 2M Guanidine-HCL solution and Endoproteinase Lysine C added (1:20 protease:GST). Protease digestion was

performed overnight at 37°C (Stone et al; Chap. 2  
in "A Practical Guide to Protein and Peptide  
Purification for Microsequencing " ed. Matsudaira,  
Academic Press). The digest was injected onto a C8  
5 Reverse Phase HPLC column (SGE 2.1 \* 100mm ) and a  
gradient of acetonitrile in 0.1% Trifluoroacetic  
acid used to elute the peptide fragments . Amino  
Acid sequence analysis of one fragment gave  
sequence corresponding to amino acids 208-222 of  
10 GST-II-27 as predicted from the nucleic acid  
sequence. This sequence has no homology with the  
published sequence for GST-I-29 or GST-III-26.

Further amino acid sequence was obtained by  
digesting the reduced and alkylated GST-II-27 with  
15 trypsin for 2.5 hours at 20°C. The peptides were  
separated by Reverse Phase HPLC ( using a Vydac C8  
4.6 \* 150mm , 300 Å pore column) and a gradient of  
acetonitrile. Three peptides were sequenced and  
these sequences corresponded to amino acids 8-19,  
20 20-38 and 52-71 of GST-II-27 as predicted from the  
nucleic acid sequence. This shows that the  
isolated cDNA clone corresponds to the GST-II-27  
subunit.

Figure 4 compares the amino acid sequences of  
25 GST-II-27, GST-I-29 and GST-III-26. An asterisk  
(\*) indicates a position in the alignment is  
perfectly conserved; a dot (.) indicates a position  
is well conserved; a tick (✓) indicates a position  
which is conserved with rat GSTs. GST-II-27 shows  
30 homology to the two known isoforms, GST-I-29 and  
GST-III-26. GST-II-27 is 57% identical with  
GST-I-29 and 41% with GST-III-26. Furthermore,  
several residues are conserved with rat GSTs.

### GENOMIC CLONE ISOLATION

The cDNA for GST-II-27 was utilised to design a gene probe for the isolation of a corresponding genomic sequence which included the promoter region.

The GST-II-27 cDNA sequence was examined to identify a specific region which does not occur in other maize GSTs or other plant/animal/viral/vector sequences, and hence would be suitable as a specific gene probe. A 208 bp sequence was isolated by PCR from the 3' end of plasmid pIJ21 and purified by acrylamide gel electrophoresis. This PCR probe was random prime labelled and used to screen  $5 \times 10^6$  recombinants from a maize genomic library (partial MboI digested DNA with an average insert size of 15kB).

The positive clones were plaque-purified with the 3' PCR probe and a 5' oligo (130 bp from the 5' end of the cDNA). These genomic clones were then mapped to identify fragments running from the 5' end of the cDNA into the promoter region. An EcoRI fragment containing around 4kB of promoter region was isolated. This fragment was subcloned into a pBS vector, designated plasmid pGIE7, which was deposited in the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, with the accession number NCIMB 40426.

Primer extension analysis (as described by Ausubel et al, 1987, Current Protocols in Molecular Biology) was used to map the transcription start point (TSP) to 16 bp from the end of the cDNA as shown in Figure 5. Sequence analysis of the promoter subclone pGIE7 revealed putative TATA and CAAT boxes -29 bp and -110 bp from the predicted



TSP respectfully. The TSP site, TATA box and intron/exon boundary regions fit the plant consensus sequences, confirming that the isolated sequence is indeed the promoter sequence.

5

#### SEQUENCING OF THE GST-II-27 PROMOTER FRAGMENT

Figure 6 represents the 5' end of the GST-II-27 gene, showing the relative position of restriction sites and the EcoRI fragment isolated above. PCR and restriction analysis of the genomic clones had indicated that two introns covering 300 bp are present in the region corresponding to the 5' end of the cDNA, and that one of the introns contains an EcoRI cleavage site. Plasmid pGIE7 contains the EcoRI-EcoRI fragment which covers the GST-II-27 promoter region plus some coding sequence. The sequence of exon 1 (underlined) matches that of the 5' end of the cDNA.

Genomic subclones pGIE7 and pGIS15 were used to sequence 5.4 Kb of the GST-II-27 gene and promoter. The majority was sequenced on both strands according to the strategy illustrated in Figure 7. Figure 8 shows the nucleotide sequence of 3.8 kb of the promoter sequence from the 5' EcoRI site to the predicted translation start point.

#### GST-II-27 PROMOTER CONSTRUCTS

Using a combination of fine mapping of promoter subclones and sequence data, appropriate restriction sites were chosen for promoter constructs. A variety of transient assay vectors for protoplast systems (pPUG) and stable transformation vectors for tobacco (pGSTTAK) and

Zea mays (pZM/RMS-3) were constructed. All recombinant plasmids were identified by hybridisation probes, their integrity and orientation checked by restriction mapping and boundaries of completed vectors analysed by sequencing.

A variety of transient and stable transformation GUS vectors were constructed using approximately 3.8 Kb of the GST-II-27 promoter. Nde I was used to cut the GST-II-27 promoter at the ATG and 4 Kb upstream. This fragment was cut with EcoRI, blunted and cloned into Sma I site of pTAK (a Bin19 based promoterless GUS construct). The GST GUS cassette from pGSTTAK was then cloned into the transient assay vector pPUG (3.8 GST promoter and GUS). The same GST GUS cassette was also cloned into a pUC derived vector containing the Bar selectable cassette giving pZM/RMS-3.

Figure 9 gives an overview of the method of vector construction. Figure 10 shows the final structure of the maize transient transformation vector, pPUG; Figure 11 shows the tobacco stable transformation vector, pGSTTAK; and Figure 12 shows the maize stable transformation vector, pZM/RMS-3.

#### PROTOPLAST TRANSIENT EXPRESSION ASSAY SYSTEM

A series of protoplast transient assay experiments using pPUG were carried out to demonstrate the 3.8 kB GST-II-27 promoter region was transcriptionally active and that expression from this promoter could be induced by herbicide safeners.

Protoplasts were isolated from maize cell suspension lines or from in vitro grown maize

leaves by a standard enzymatic digestion, sieving and washing procedure. Transformation was carried out by means of a modified PEG-mediated uptake method (based on Negrutiu et al, 1987, Plant Mol Biol, 8:363-373), using 25  $\mu$ g DNA/ $0.4 \times 10^5$  protoplasts. Chemical was added to the protoplast culture medium at 3-300 ppm for induction of GST followed by incubation at 25°C in the dark. Viability of the treated protoplasts was assessed after 24 hours prior to preparation of extracts. GUS expression was measured by means of a fluorimetric GUS assay (Jefferson, 1987, Plant Mol Biol Rep, 5:387-405).

A summary of results is shown in Figure 13 which gives the concentration of pPUG expression in protoplasts after 19 hours incubation with or without safener. Results demonstrate that the 3.8 Kb promoter of GST-II-27 is capable of controlling GUS levels in an inducible manner in a number of protoplast systems.

#### STABLE TRANSFORMATION EXPERIMENTS IN TOBACCO

The Bin19 vector pGSTAK containing 3.8 Kb of the GST-II-27 promoter 5' to the GUS reporter gene and nos terminator was used to generate transgenic tobacco using Agrobacterium Ti plasmid technology as described by Bevan (1984, Nucleic Acids Research, 12:8711-8721). Transformants produced by this procedure were used in leaf painting experiments in which 10 mg of formulated safener was applied to a 10 cm<sup>3</sup> region of leaf. A second application was made to the same area 48 hours later, and the tissue was harvested after a further 24 hours. Treated and untreated leaf tissue was

assayed for GUS activity using the procedure of Jefferson (1987, Plant Mol Biol Rep, 5:387-405).

A range of transformants were examined using this method. As shown in Figure 14, safener application had elevated GUS expression by 100 fold in some cases. This clearly demonstrates that the monocotyledonous GST-II-27 promoter can regulate gene expression in a dicotyledonous species in an inducible manner.

#### STABLE TRANSFORMATION EXPERIMENTS IN MAIZE

The GST GUS vector RMS 3 was used to generate transgenic maize plants using particle bombardment (Gordon-Kamm et al, 1990, Plant Cell, 2:603-618). When leaf tissue was painted with herbicide safener as described above, inducible GUS activity was observed (as shown in Figure 15).

#### GST-II-27 INDUCTION IN VARIOUS TISSUES

The induction of GST-II-27 in several tissues of developing maize was studied to determine how the promoter works in its natural environment.

Formulated R-29148 (2,2,5-trimethyl-3-(dichloroacetyl)-1,3-oxazolidine) or formulation alone were applied as a root drench to maize plants (400 mg R-29148/plant) and after a period of time crude protein extracts were prepared from samples of mature and immature leaf, root, stem and tassel. Western blot analysis was carried out using these extracts and the anti-GST-II-27-sera or the anti-GST-I-29-sera. The results show that the 29 kD subunit (GST-I-29) is constitutively expressed in all the tissues tested and was inducible in all tissues by safener application. The 27 kD subunit,

specific to GST-II, was only constitutively expressed in root tissue. Subsequent to the root drench application of R-29148, GST-II expression was detected in all tissues tested. This induction was not detected in the plants treated with formulation lacking R-29148. Figure 16 shows the western blots allowing tissue localisation of the GST-II 27 kD subunit in untreated (control) and safener treated maize mature leaf (2), immature leaf (3), stem (4), root (5) and tassel (6). The blots also include markers (lane 1) and pure GST-II (lane 7).

Results of these experiments suggest that the promoter of the invention may be used to control gene expression in a variety of transgenic tissues.

FIELD TRIAL SHOWING GST-II-27 INDUCTION IN TASSEL TISSUE BY SAFENER APPLICATION.

A field trial was performed to examine the expression of GST isoforms in developing maize tassels subsequent to the external application of chemical safeners. The trial demonstrated the statistically significant induction of GST-II under field conditions.

Two safeners were used in the field trial: R-25788 (N,N-diallyl-2,2-dichloroacetamide) and R-29148 (2,2,5-trimethyl-3-(dichloroacetyl)-1,3-oxazolidine), and these were applied in a formulation of cyclohexanone, synperonic NPE1800 and Tween 85 or a formulation of Solvesso 100, Synperonic NB13 and Phenylsulphonate.

Safener was applied by a spray application using overhead spraying (30 cm above plants) at

four application rates (three replications per rate) as follows:

5           X    2.58 Kg/Ha  
          2X    5.16 Kg/Ha  
          4X    10.32 Kg/Ha  
          8X    20.64 Kg/Ha).

The control treatments involved spraying formulation blanks 2F (treatment 2X without safener) and 8F (treatment 8X without safener).

10           After a period of time, the tassels were harvested and split into three sizes: pre-meiotic, meiotic and post-meiotic floret tissue. Crude protein extracts were prepared from floret tissue and stored at -70°C.

15           Western blot analysis was performed using these crude extracts and the antisera raised in sheep against the GST-I-29 or the GST-II-27 subunits. Antisheep antisera linked to Horseradish peroxidase was used as the secondary antisera and  
20           detection was achieved using Enhanced Chemical Luminescence (Amersham International PLC). The levels of GST-II-27 present in the crude extract was quantified by running standards of purified GST-II-27 in lanes of the SDS-PAGE gel adjacent to  
25           the crude extract samples and performing densitometric analysis on the blots.

          The levels of GST-II induction was also determined by separating the GST isoforms by FPLC ion exchange chromatography and assaying for the  
30           presence of GST using CDNB as the substrate.

          Some results of the field trials are given in Figures 17 and 18. Figure 17 gives the overall mean values for treatment with R-25788 and R-29148. It is obvious that the presence of the

inducing chemical ( treatments X, 2X, 4X, 8X) causes an increase in the amount of the GST-II enzyme under field conditions when compared to the control plants (treatments 2F and 8F). Figure 18 gives the amount of GST-II from 2F field trial tassel samples (large, medium and small), estimated by western analysis. When compared to the 2X treated samples, it is obvious that the presence of inducing chemical causes an increase in GST-II over the basal level.

These results show that the safeners R-29148 and R-25788 can be used to induce expression of GST-II under field conditions. These data, taken with the results from the studies with transgenic plants containing the GST-II-27 gene promoter, suggest that said promoter will provide a useful and efficient mechanism for controlling gene expression in transgenic plants under field conditions.

#### PRODUCTION OF HERBICIDE RESISTANT TRANSGENIC PLANTS EXPRESSING THE GST-II ENZYME

The nucleotide sequence of a cDNA encoding GST-I-29 was published by Wiegand et al. in Plant Mol Biol, 1986, 7:235-243. Using this sequence, a specific oligonucleotide probe was designed and used to isolate a full length cDNA (plasmid pIJ4) encoding GST-I-29 from a safener induced seedling root cDNA library. Isolation of the cDNA encoding GST-II-27 (as shown in Figure 2) has already been described. The full length cDNAs may be incorporated into Bin19-based vectors under the control of the 35S CaMV promoter.

Figure 19 shows how these constructs may be prepared. The full length GST-I-29 coding region and the full length GST-II-27 sequence are isolated from pIJ4 and from pIJ21 respectively by digestion with EcoRI. These fragments are filled in using Klenow/T4 polymerase to produce blunt ends. The blunt-ended fragments are then ligated 3' to the 35S CaMV promoter of pJRII at the SmaI cloning site. Recombinants containing cDNA inserts in the correct orientation are be selected using restriction mapping analysis.

Tobacco plants may be transformed with vectors containing either the CaMV35S-GST-I-29 or the CaMV35S-GST-II-27 cassette using the protocols already described. Transformants expressing the respective GST-II subunits (29 kD or 27 kD) may be determined by Western blotting analysis with subunit specific antisera (as already described). Such transformants may be crossed to produce progeny expressing both GST-I-29 and GST-II-27, resulting in a herbicide resistant phenotype.



International Application No: PCT/

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**MICRO ORGANISMS**Optional Sheet in connection with the microorganism referred to on page 2, line 18 of the description \***A. IDENTIFICATION OF DEPOSIT \***Further deposits are identified on an additional sheet ☒

Name of depository institution \*

National Collections of Industrial and  
Marine Bacteria

Address of depository institution (including postal code and country) \*

23 St Machar Drive, Aberdeen,  
Scotland, AB2 1RY, UK.

Date of deposit \*

14 June 1991

Accession Number \*

NCIMB 40426

**B. ADDITIONAL INDICATIONS \*** (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited micro-organism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue or such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE \*** (if the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS \*** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later \* (Specify the general nature of the indications e.g., "Accession Number of Deposit")

**E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)**The Patent Office  
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Gwent NP9 1RH

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J.R. Lloyd Thomas  
(Authorized Officer)☐ The date of receipt (from the applicant) by the International Bureau is:

was

(Authorized Officer)

International Application No: PCT/

<b>MICROORGANISMS</b>	
Optional Sheet in connection with the microorganism referred to on page <u>2</u> , line <u>33</u> of the description *	
<b>A. IDENTIFICATION OF DEPOSIT *</b>	
Further deposits are identified on an additional sheet <input checked="" type="checkbox"/> :	
Name of depository institution * National Collections of Industrial and Marine Bacteria	
Address of depository institution (including postal code and country) * 23 St Machar Drive, Aberdeen, Scotland, AB2 1RY, UK	
Date of deposit *	Accession Number *
19 April 1991	NCIMB 40413
<b>B. ADDITIONAL INDICATIONS *</b> (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, a sample of the deposited micro-organism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).	
<b>C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *</b> (if the indications are not for all designated States)	
<b>D. SEPARATE FURNISHING OF INDICATIONS *</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
<b>E. <input checked="" type="checkbox"/> This sheet was received with the international application when filed (to be checked by the receiving Office)</b> <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 45%;"> <p>20/GB The Patent Office Cardiff Road, Newport Gwent NP9 1RH</p> </div> <div style="width: 45%; text-align: right;"> <p><i>J.R. J. L. Thomas</i> (Authorized Officer)</p> </div> </div>	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau **  <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;">was</div> <div style="width: 45%; text-align: right;">(Authorized Officer)</div> </div>	

CLAIMS

- 1 A genomic DNA sequence encoding the gene  
promoter for the 27 kD subunit of  
glutathione-S-transferase, isoform II,  
containing the nucleotide sequence shown in  
5 Figure 8 herewith and variants of the said  
sequence as permitted by the degeneracy of the  
genetic code.
- 2 A vector containing the genomic DNA sequence  
10 as claimed in claim 1.
- 3 A chemically switchable gene construct which  
includes the promoter sequence as claimed in  
claim 1 operatively linked to a foreign gene  
15 or a series of foreign genes whereby  
expression of said foreign gene or said series  
of genes may be controlled by application of  
an effective exogenous inducer.
- 20 4 Plants transformed with a gene construct as  
claimed in claim 3.
- 5 5 Plants as claimed in claim 4 which are  
monocotyledons.  
25
- 6 6 Plants as claimed in claim 4 which are  
dicotyledons.
- 7 7 Plants as claimed in any of claims 4 to 6 in  
30 which presence of the foreign gene or series  
of genes affects male fertility.

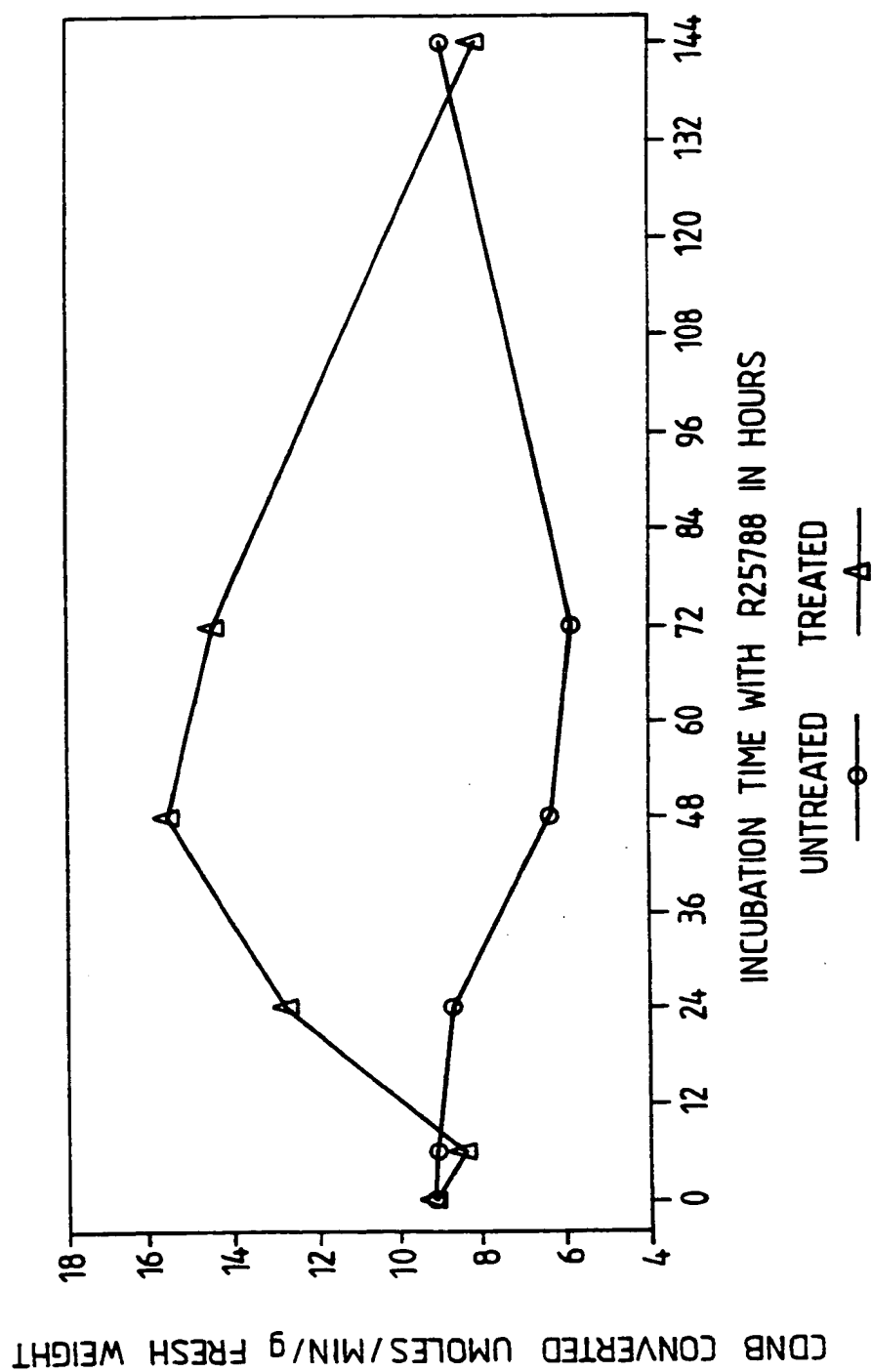
- 8      Glutathione-S transferase, isoform II, 27 kD  
subunit having substantially the same amino  
acid sequence as shown in Figure 4 herewith.
- 5      9      A cDNA sequence encoding the subunit as  
claimed in claim 8, having the nucleotide  
sequence shown in Figure 2 herewith and  
variants of the said sequence as permitted by  
the degeneracy of the genetic code.
- 10      10      A method whereby the sequence as claimed in  
claim 9 is utilised as a gene probe for the  
isolation of a corresponding genomic sequence  
which encodes the glutathione-S-transferase,  
15      isoform II, 27 kD subunit and includes the  
promoter region.
- 20      11      A method to produce a herbicide resistant  
transgenic plant which comprises the  
incorporation of DNA encoding one or more GST  
polypeptides into the plant such that a  
glutathione-S-transferase enzyme is expressed.
- 25      12      A method as claimed in claim 11 in which DNA  
encoding the GST-I 29 kD subunit is  
incorporated into the plant.
- 30      13      A method as claimed in claim 11 in which DNA  
encoding the GST-II 27 kD subunit is  
incorporated into the plant.

- 14      A method as claimed in claim 11 in which DNA  
encoding the GST-I 29 kD subunit and the  
GST-II 27 kD is incorporated into the plant.
- 5      15      A method as claimed in claim 11 in which DNA  
encoding the GST-I 29 kD subunit is introduced  
into a first plant, DNA encoding the GST-II 27  
10      kD subunit is introduced into a second plant,  
and the first and second plant are crossed to  
produce herbicide resistant progeny.
- 16      A method as claimed in claim 11 in which DNA  
encoding the GST-III 26 kD subunit is  
15      incorporated into the plant.
- 17      A method as claimed in claim 11 in which DNA  
encoding the GST-I 29 kD subunit and the  
GST-III 26 kD subunit is incorporated into the  
20      plant.
- 18      A method as claimed in claim 11 in which DNA  
encoding the GST-I 29 kD subunit, the GST-II  
27 kD subunit and the GST-III 26 kD subunit is  
25      incorporated into the plant.
- 19      A method as claimed in any of claims 11 to 18  
in which the DNA encoding the GST polypeptide  
is under the control of an constitutive  
30      promoter.

- 20 . A method as claimed in any of claims 11 to 18  
in which the DNA encoding the GST polypeptide  
is under the control of an inducible promoter.
- 5 21 A method as claimed in claim 20 in which the  
inducible promoter is the GST-II-27 promoter.
- 22 A herbicide resistant transgenic plant  
produced by the method as claimed in any of  
10 claims 11 to 21.
- 23 A plant as claimed in claim 22 in which  
herbicide resistance is constitutive.
- 15 24 A plant as claimed in claim 23 which is maize.
- 25 A plant as claimed in claim 22 in which  
herbicide resistance is inducible.
- 20 26 Progeny and seed of plants as claimed in claim  
22.

1/24

FIG. 1



2/24

FIG. 2 (1/3)

	10	20	30	40	50
1	CCAGCTGCTG	ATCTTGATCC	TGCACCCCGA	GCCGTACACA	AGAGCTAGTC
	GGTCGACGAC	TAGAACTAGG	ACGTGGGGCT	CGGCATGTGT	TCTCGATCAG
51	GGTAGAACTT	GCAGGAGCGG	AGCAGAACTA	AGTGCAGAGA	ACAGGACATA
	CCATCTTGAA	CGTCCCTCGCC	TCGTCTTGAT	TCACGTCTCT	TGTCCTGTAT
101	TGGCTACGCC	GGCGGTGAAG	GTTACGGGT	GGGCTATCTC	GCCGTTTCGTA
	ACCGATGCGG	CCGCCACTTC	CAAATGCCCA	CCCGATAGAG	CGGCAAGCAT
151	TCGCGGGCTC	TGCTGGCCCT	GGAGGAGGCC	GGCGTCGACT	ACGAGCTCGT
	AGCGCCCGAG	ACGACCGGGA	CCTCCTCCGG	CCGCAGCTGA	TGCTCGAGCA
201	CCCCATGAGC	CGCCAGGACG	GCGACCACCG	CCGCCCGGAG	CACCTCGCCA
	GGGGTACTCG	GCGGTCCTGC	CGCTGGTGGC	GGCGGGCCTC	GTGGAGCGGT
251	GGAACCCCTT	CGGGAAGGTG	CCGGTGCTCG	AGGATGGCGA	CCTCACGCTC
	CCTTGGGAAA	GCCCTTCCAC	GGCCACGAGC	TCCTACCGCT	GGAGTGCCGAG
301	TTCGAATCAC	GTGCGATCGC	GAGGCATGTT	CTCCGGAAGC	ACAAGCCGGA
	AAGCTTAGTG	CACGCTAGCG	CTCCGTACAA	GAGGCCTTCG	TGTTCCGGCCT
351	GCTGCTGGGC	GGCGGCAGGC	TGGAGCAGAC	GGCGATGGTG	GACGTGTGGC
	CGACGACCCG	CCGCCGTCCG	ACCTCGTCTG	CCGCTACCAC	CTGCACACCG
401	TGGAGGTGGA	GGCCCCACCAG	CTGAGCCCCG	CGGCGATCGC	CATCGTGGTG
	ACCTCCACCT	CCGGGTGGTC	GA CT CGGGCG	GCCGCTAGCG	GTAGCACCAC



3/24

FIG. 2 (2/3)

401 TGGAGGTGGA GGCCACCAG CTGAGCCCGC CGCGGATCGC CATCGTGGTG  
ACCTCCACCT CCGGGTGGTC GACTCGGGCG GCCGCTAGCG GTAGCACAC

451 GAGTGCGTGT TCGCGCCGTT CCTGGGCGCG GAGCGCAACC AGGCGGTGGT  
CTCAGGCACA AGCGCGGCAA GGACCCGGCG CTCGCGTTGG TCCGCCACCA

501 GGACGAGAAC GTGAGAAGC TCAAGAAGT GCTGGAGGTG TACGAGGCGC  
CCTGCTCTTG CACCTCTTCG AGTTCTTCCA CGACCTCCAC ATGCTCCGCG

551 GGCTGGCCAC GTGCACGTAC CTCGCCGGCG ACTTCTCTAG CCTCGCCGAC  
CCGACCGGTG CACGTGCATG GAGCGGCCCG TGAAGGAGTC GGAGCGGCTG

601 CTCAGCCCCCT TCACCATCAT GCACTGCCCTC ATGGCCACCG AGTACGCCGC  
GAGTCGGGGA AGTGGTAGTA CGTGACGGAG TACCGGTGGC TCATGCGGCG

651 TCTCGTCCAT GCGTCCCCG ACGTCAGCGC CTGGTGGCAG GGCCTCGCCG  
AGAGCAGGTA CGCGAGGGCG TGCAGTCGCG GACCACCGTC CCGAGCGGC

701 CGCGCCCGGC GGCCAACAAG GTGGCGCAGT TCATGCCCGT CGCGCCCGGA  
GCGGGGCGG CCGGTGTTC CACCGCGTCA AGTACGGCCA GCCGCGGCCT

751 GCGCCCCAAG AACAGGAGTG ACGATGAAGC GATCGAAGCG ACTTGTGTG  
CGCGGGTTCC TTGTCCTCAC TGCTACTTCG CTAGCTTCGC TGAACACAAC

SUBSTITUTE SHEET

FIG. 2 (3/3)

4/24

801 TTGTGCTTGA TTAGTTAATT GGAAACCTTC TCACTCATCT AGTCCATCAT  
AACACGAACT AATCAATTAA CCTTGGAG AGTGAGTAGA TCAGGTAGTA

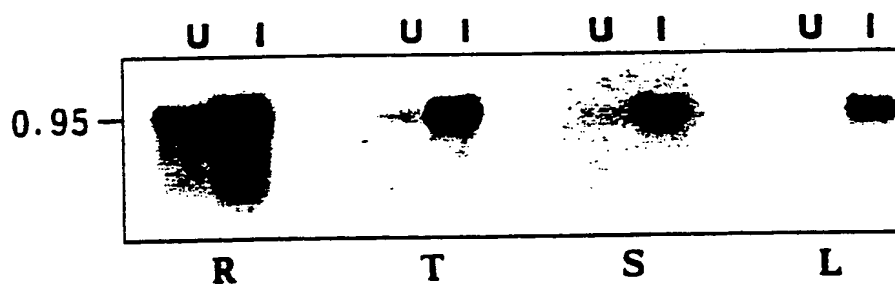
851 GGTGCCCTGCT TTTCTTTATA CTATTTGTCT TAAATTTGCT GCTTCTCTCCA  
CCACGGACGA AAAGAAATAT GATAAACAGA ATTAAACGA CGAAAGAGGT

901 CGGAATAATA GTAGAGATTT GGAAATGTAA TGTATTTATC AAAAAAAA  
GCCTTATTAT CATCTCTAAA CCTTACATT ACATAAATAG TTTTTTTTTT

951 AAAA  
TTTT

5/24

FIG. 3



SUBSTITUTE SHEET

## FIG. 4

GSTII	MATPAVKVYGWAISPFVSRALLALEEAGVDYELVPMRQDGDHRRPEHLA	50
GSTIPEP	MA--PMKLYGAVMSWNLTRCATALEEAGSDYEIVPINFATAEHKSPEHLV	48
GST3PEP	MA--PLKLYGMPLSPNVVRVATVLTNEKGLDFEIVPVDLTTGAHKQPDFLA	48
	** ..** . * . * . * . * . * . * . *	
	✓	
GSTII	RNPFCKVPVLEDDLTLEFESRAIARHVLRRKKHKE---LLGGRLQETAMV	97
GSTIPEP	RNPFQVQPALQDGLYLFESRAICKYAARKNKPE---LLREGNLEEAAMV	95
GST3PEP	LNPFQIIPALVDGDEVLFESRAINRYIASKYASEGTDLLPAT--ASAACL	96
	*** . . . * * * * * * * * * * * * * * *	
	✓	
GSTII	DVWLEVEAHQLSPPAIAIVVECVFAPFLGRERNQAVVDENVEKLKKVLEV	147
GSTIPEP	DVWIEVEANQYTAALNPILFQVLISPLMGTTDQKVVDENLEKLKKVLEV	145
GST3PEP	EVWLEVESHHFHPNASPLVFQLLVRPLGGAPDAAVVEKHAEQLAKVLDV	146
	. ** . * * * . . . . . * * * . . . * * * * * *	
	6/24	
GSTII	YEARLATCTYLAGDFLSLADLSPFTIMHCLMAT--EYAALVHALPHVSAW	195
GSTIPEP	YEARLTKCKYLAGDFLSLADLNHVSVTLCLFAT--PYASVLDAYPHVKAW	193
GST3PEP	YEAHLARNKYLAGDEFTLADANH--ALLPALTSARPPRPGCVAARPHVKAW	195
	*** . * . * * * * * * * * * * * * * * *	
	✓	
GSTII	WQGLAARPAANKVAQFMPVGAGAPKEQEX	224
GSTIPEP	WSGLMERPSVQKVAALM-----KPSA	214
GST3PEP	WEAIAARPAFQKTVAIPLP--PPPSA	222
	* . . . * * * . . . . . *	

7/24

*FIG. 5* (1/2)

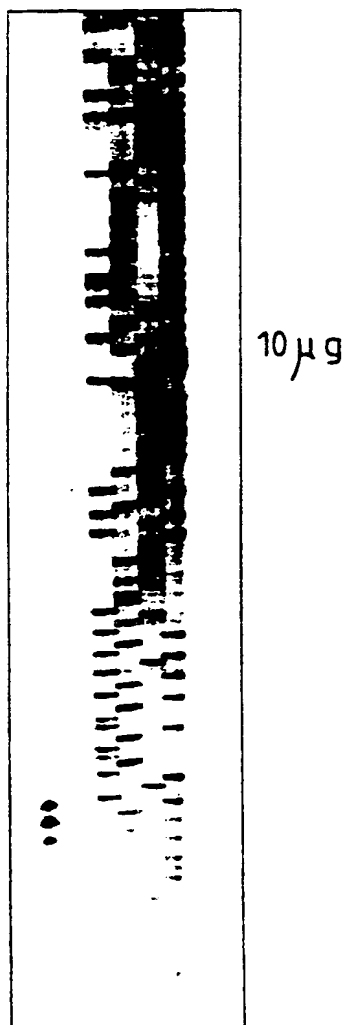


FIG. 5<sub>(2/2)</sub>

3539 tcctccgcgaccaaatacacattggctcttctagcaccttcttcttccaagactccaa  
[AAT Box

2

tccctcccgtcgaccaatacacacttggctcttcttagcacctcttccctccaagactccaa

3600 Tcccccaaccagaaccagcgccagctctaacgtcacctctgattctctctctctct

7

tccccaacaccagaaccagcgccagctctaacgtcacctctgatttcttctctctctct  
3661 attgctagctgctttatTATA BoxTSP

7

attgctagctgctttattataagtagcagctgcaggcaggcaggtgcacacaccatcc  
PRIMER PI PRIMER GST PRIM

3722 aattCCAGCTGCTGATCTTGATCCTGCACCCCGGCCGTACACAAGAGCTAGTCGGTAGAA  
|||||  
CCAGCTGCTGATCTTGATCCTGCACCCCGGCCGTACACAAGAGCTAGTCGGTAGAA

7

aaattCCAGCTGCTGATCTTGATCCTGCACCCCGAGCCGTACACAAGAGCTAGTCGGTAGAA

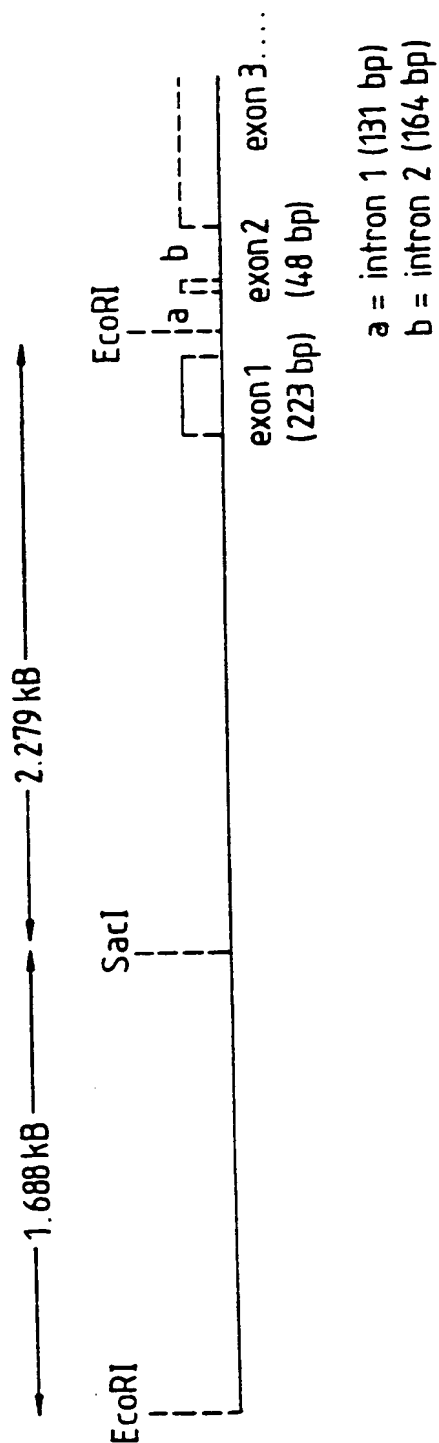
3783 CTTGCAGGAGCGGAGCAACTAAGTCAGAGAACAGGACATATGGCTACGCCCGCGGTGA

TRANSLATION START

64 CTTGCAGGCGGAGCAGAACTAAGTGCAGAGAACAGGACATATGGCTACGCCCGCGGTGA  
|||||  
CTTGCAGGCGGAGCAGAACTAAGTGCAGAGAACAGGACATATGGCTACGCCCGCGGTGA

9/24

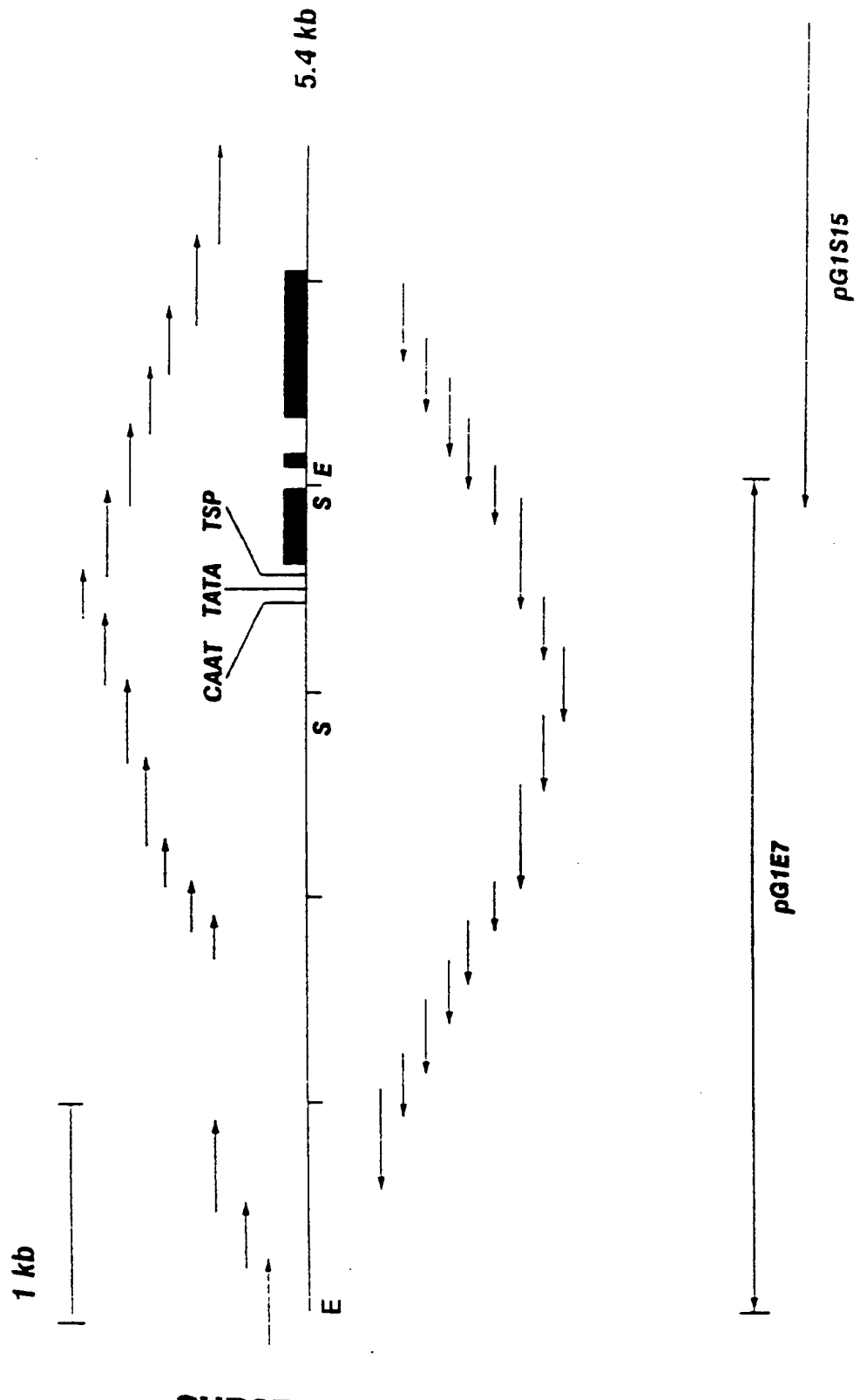
FIG. 6



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10/24

FIG. 7





11/24

FIG. 8 (1/3)

iatccaaatatatgatgattgtgtcctagtgagaaactaaatatatactagcgaaaaaaccttc  
:agtcataagtgatgggcataatagaaaaataaaacatctcaagactccaaactagtcataagcttcta  
:cacaacttcaaacacttcatgccaaagatcatggatttttttttgcctaagacaaaactagaat  
:gaaaagaactaactcatcacatatantatggcatcacaaaaaaatgacacatatatgatACTAT  
TCACACAGGCCCTTCAGTTCTAGAACAAAGTCAGATCGATgtgtgggtatgcatgtctaatatttact  
jgttgatatgcatgggcgttcattcagaatcagtttcacacagtttatcgcacttctgtttacaaaac  
tggatttcatgtctgtactggctacatgcgtaaagatcaacttgtctaatctaggtgcatcctcctt  
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aggaaactgatgtgcaacaacAAAACTAAATAGGAAGGaaacacaagtccaaatgtataataattg  
caccatagtgcaaaagaaaccaaataatactgcagagaaaaacttcctagtcagtcatgtaaagtgtATGGACATAT  
GAAATAAAACATCTCAAGACTCCCAATAACAGGCTCAAGCTAACTAGTCATGGCTTTAAACCTTCATGAT  
CAAACTAGTCACAACTTTAAACATTTTCATGCCAACAAAGATCATGGATGGTGTTTTTCCTAGGGAA  
AGCTAGAAATGAGAAAGACCTAACTCAGCATACATATCAGGATAGTATCGTATAGACACGTATATGATA  
TATATCAGCGCAGCCGTTCAATTTCTAGAACAAATGCAGATTGATCTGTGAATATGCATGTCTCATATTT  
ACTAGGTTGGATGGACTGAATCCCGTGAACAAACAATTTATTCACAAAgtttctgcatgaataatc  
caaatccaataatcactctcgttgataaaaaaatgcaaccaacagtttaaccagaagtgaatatagaac  
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ttgtttaccatcaatttcaagtacacagtaacaagaacagtacagctagaattgagcatgtgagtATT  
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13/24

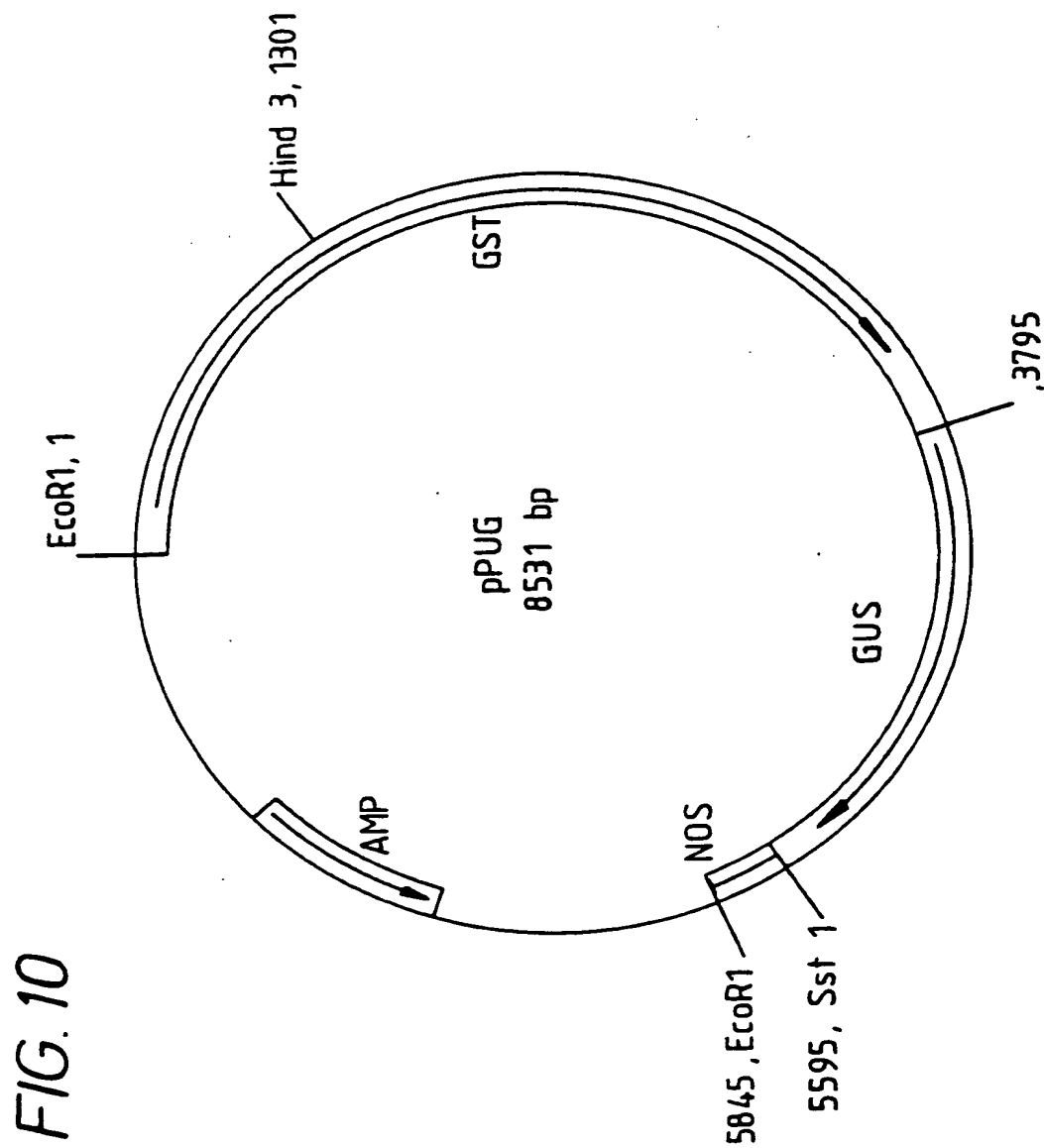
FIG. 8 (3/3)

AAAAATAACAACCTCTCCTGAAAGTGTCGGTGTGAAGCCGAGAAATCCCTTTTCATTTTCGGTGACG  
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 CGCAAGTATCGGTAGGCATTTTAAACTGAAACCCTAACTAAACCCGAAATAGACCAAAATGTTGGTTT  
 TTCGGGTTTTTGGGTTTCGGATTTCGGTTTCTAAATATGCTATATTTTAGGGTATAGGTTCCGGTTCAGTT  
 CTAACCTTTAAACCTGAATAGACGAATAACCCGAAATATAAAATCTCTTAATATGTGATGATATTA  
 TATATGATTTATGAACCTTATTAACCGAAATAATGATACCATCCTAACGATAGTATATATCTATGTA  
 GCTATTTTATAGTCACCTTGTGTAATAATAGTACTTCCAATTAATCAAGTGATATATTTTAACA  
 AAGATACTAGCCCTCTCTACTATTGTAGTATATTCCGGTGCACCCGAATAGACCGAACCGAAATTGTAAGTC  
 ATTCAGGTTTCGGTTCCCTAAAATTATTTTAAATAATTTGGTCTCATATTTTCAGAAATCCGAAATTTTCATA  
 ATCCAAATAGACCGAACCAAAATaCGCTAATAGACCGAAATAACTAGCGTACTCgCAAGTcGCACCCAC  
 AGCCTGCTGCGTGCGTAAGCGAGGACGTCACGCGTTCTCCCTCCCGTCGACCAATAACACTTGGTCTTC  
 AGCACCTTCTCTCTCCCAAGaCTCCAAATCCCCCAACCAAGACCGGCCagCTctAACGTACACCT  
 TGATTCTCTCTCTCTCTATTTGCTAGCTGCTTTATATAAGTAGCAGCTGCAGCAGGAGCTGCA  
 ACACCAATCCCAATTCAGCTGCTGATCTTGATCTCTGACCCCGAGCCGTACACAAGAGCTAGTCGGTAG  
 ACTTGCAAGGAGCGGAGcAGAACTAAGTGCAGAGAACAGGACATATG

— translation start point

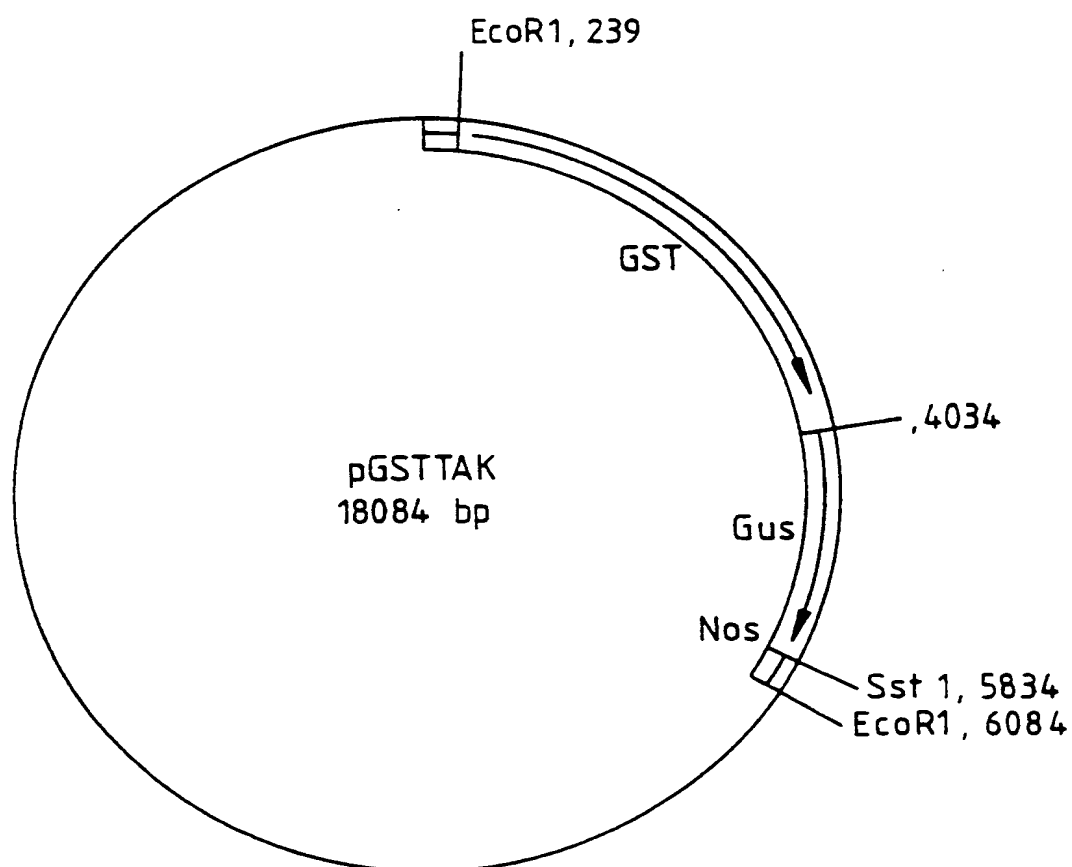
SUBSTITUTE SHEET



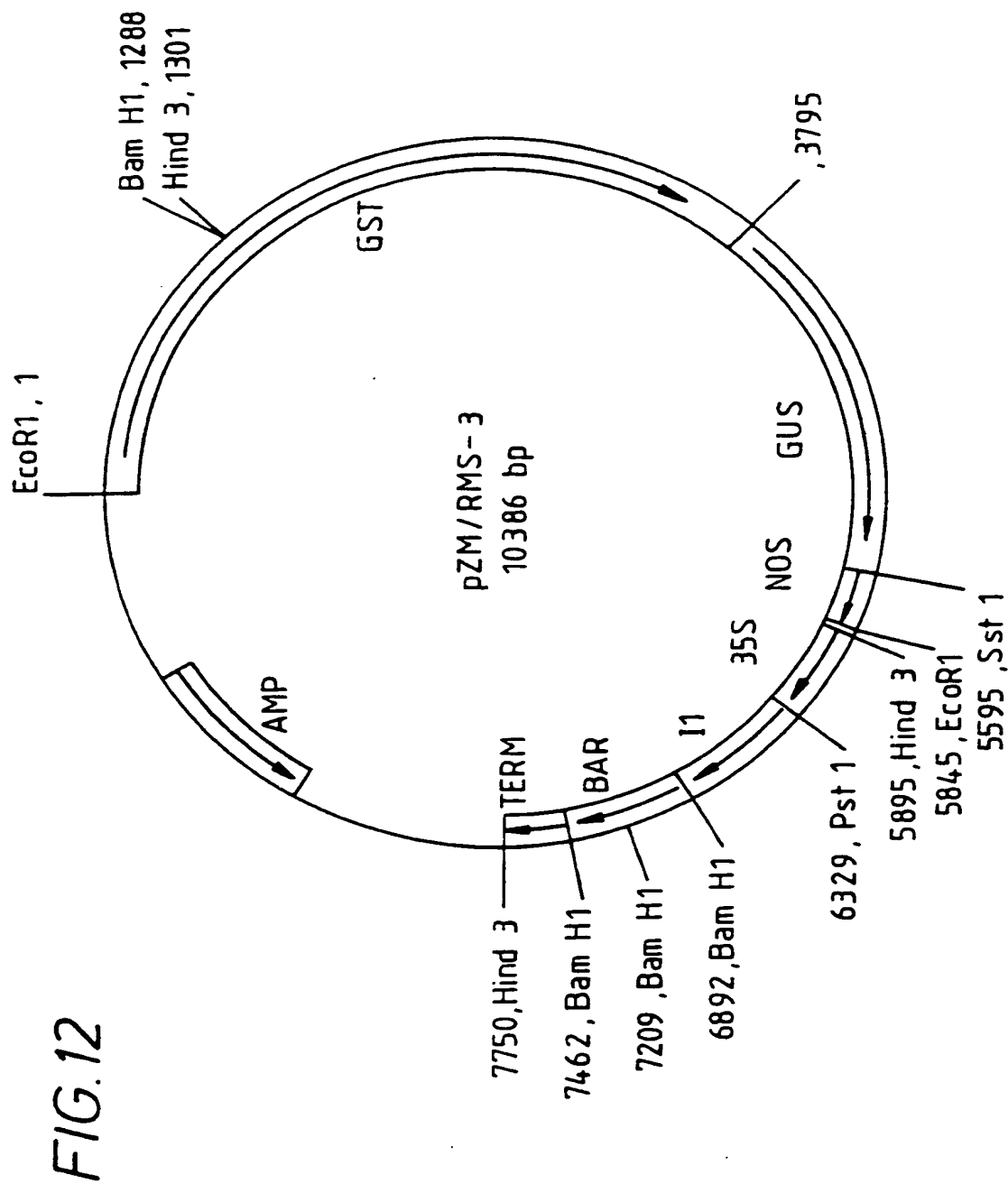


16/24

FIG. 11



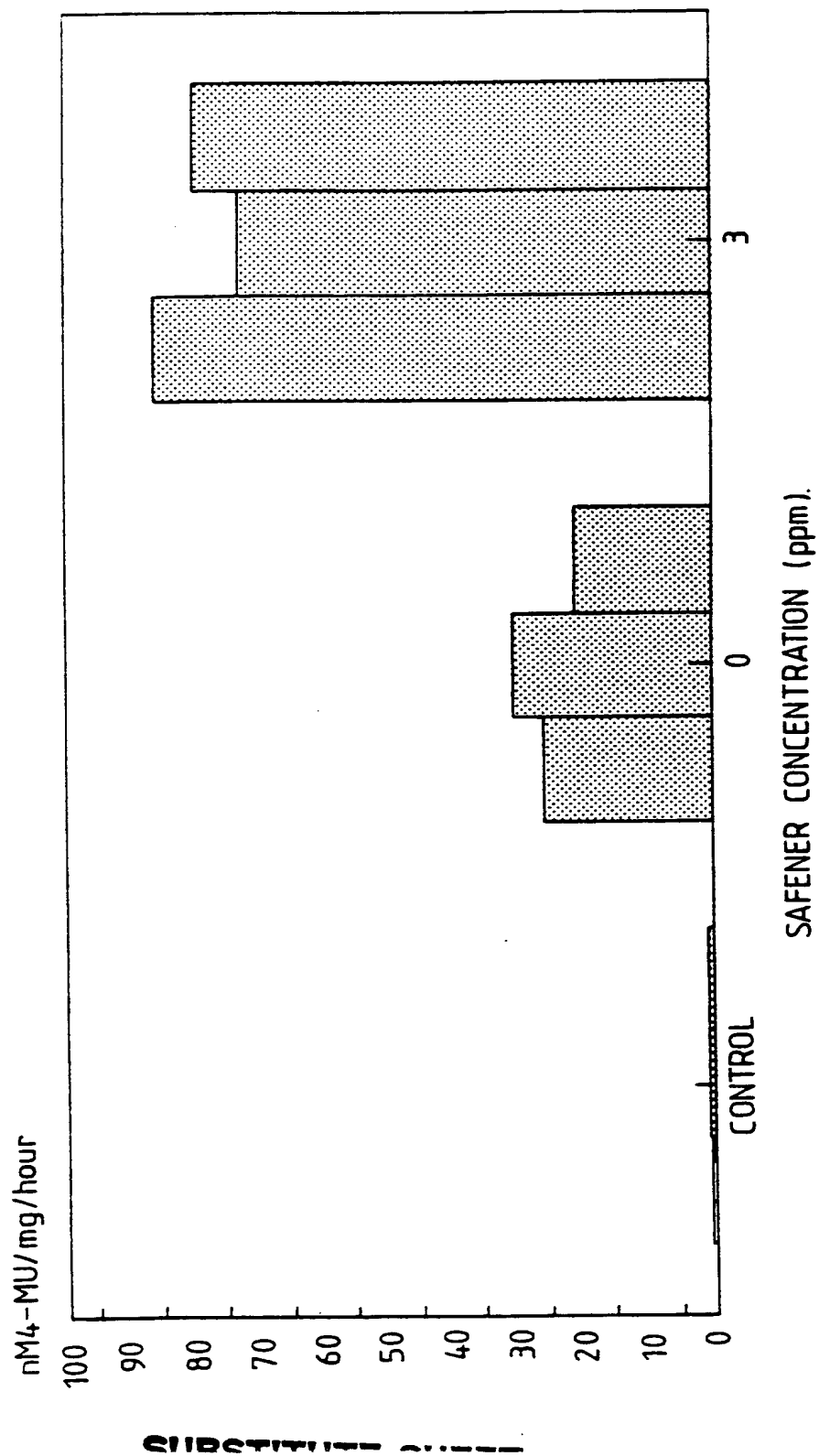
17/24



SUBSTITUTE SHEET

18/24

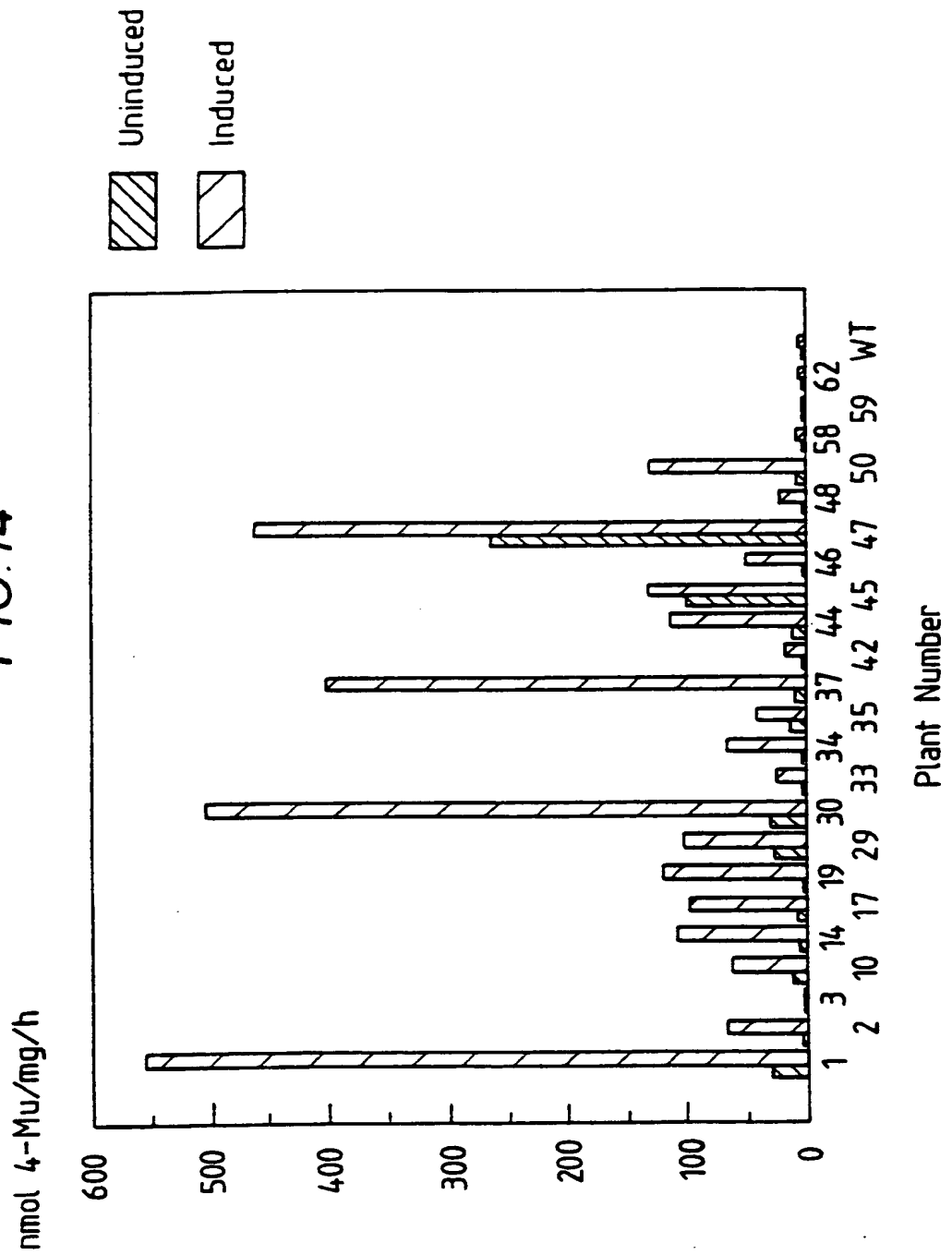
FIG. 13





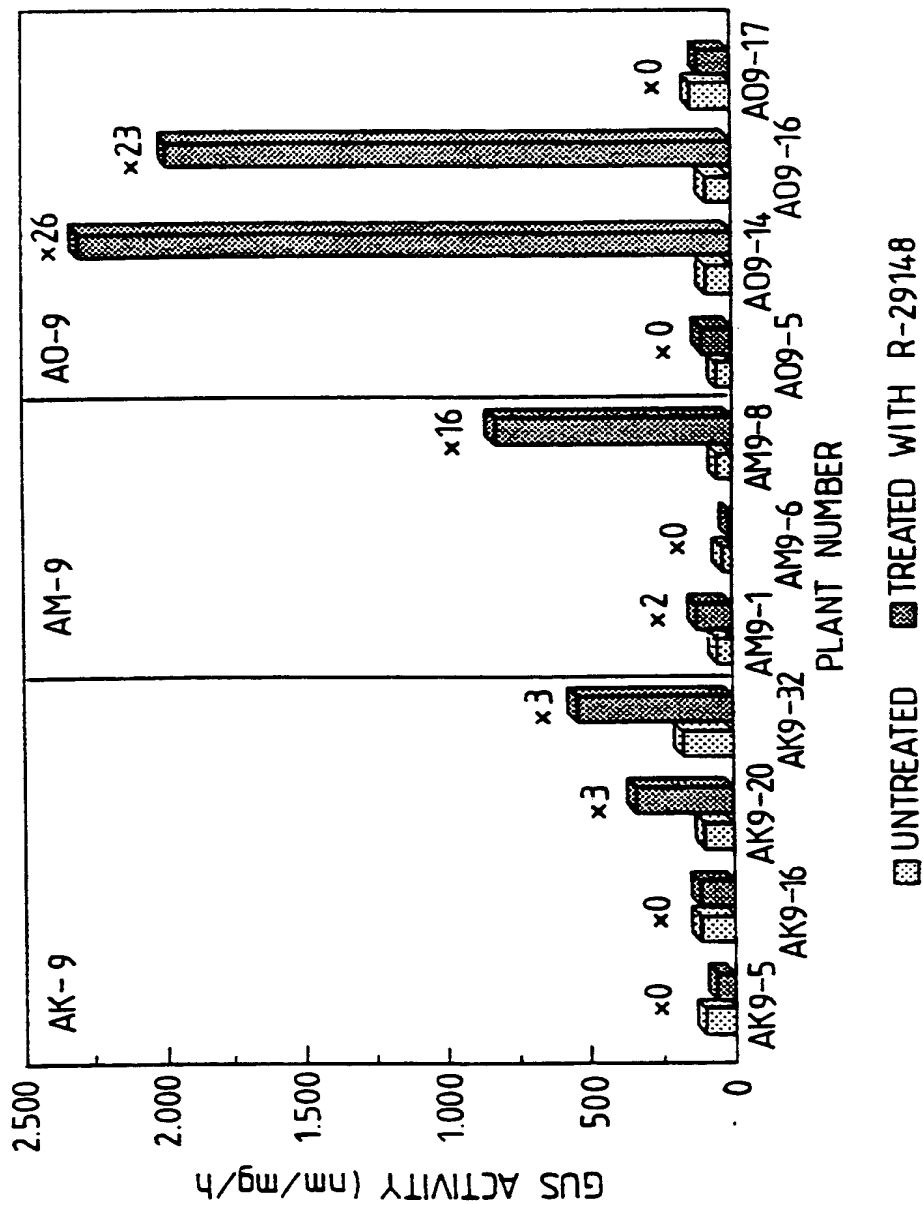
19/24

FIG. 14



20/24

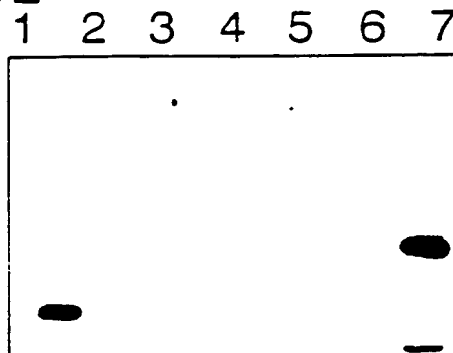
FIG. 15



21/24

*FIG. 16*

CONTROL



SAFENER TREATED

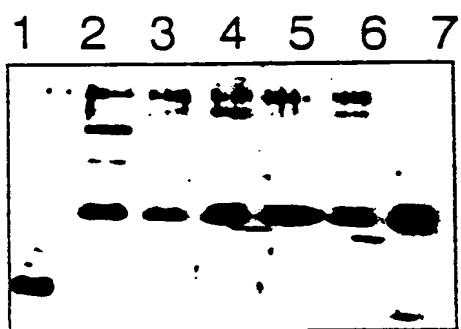
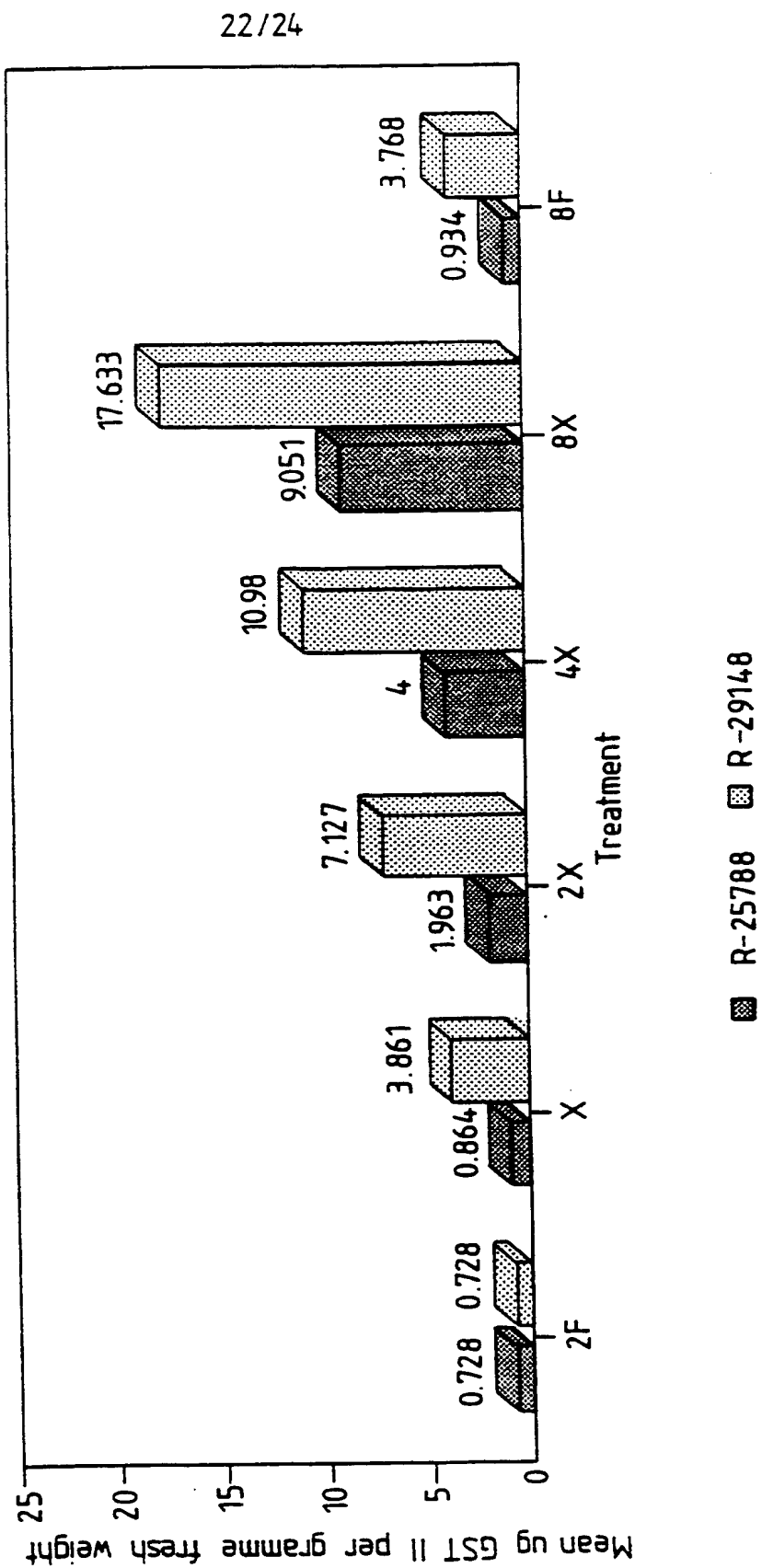
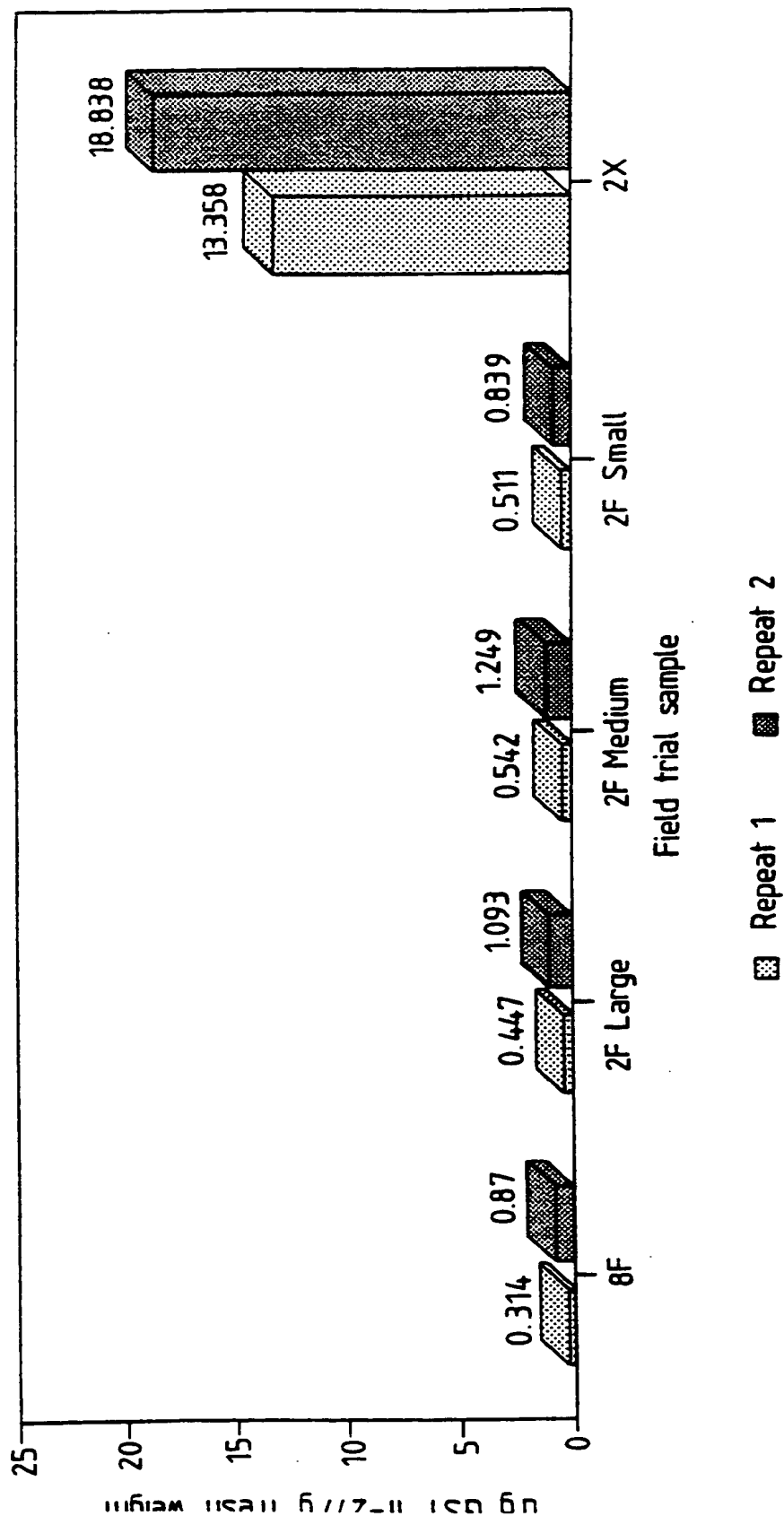
**SUBSTITUTE SHEET**

FIG. 17



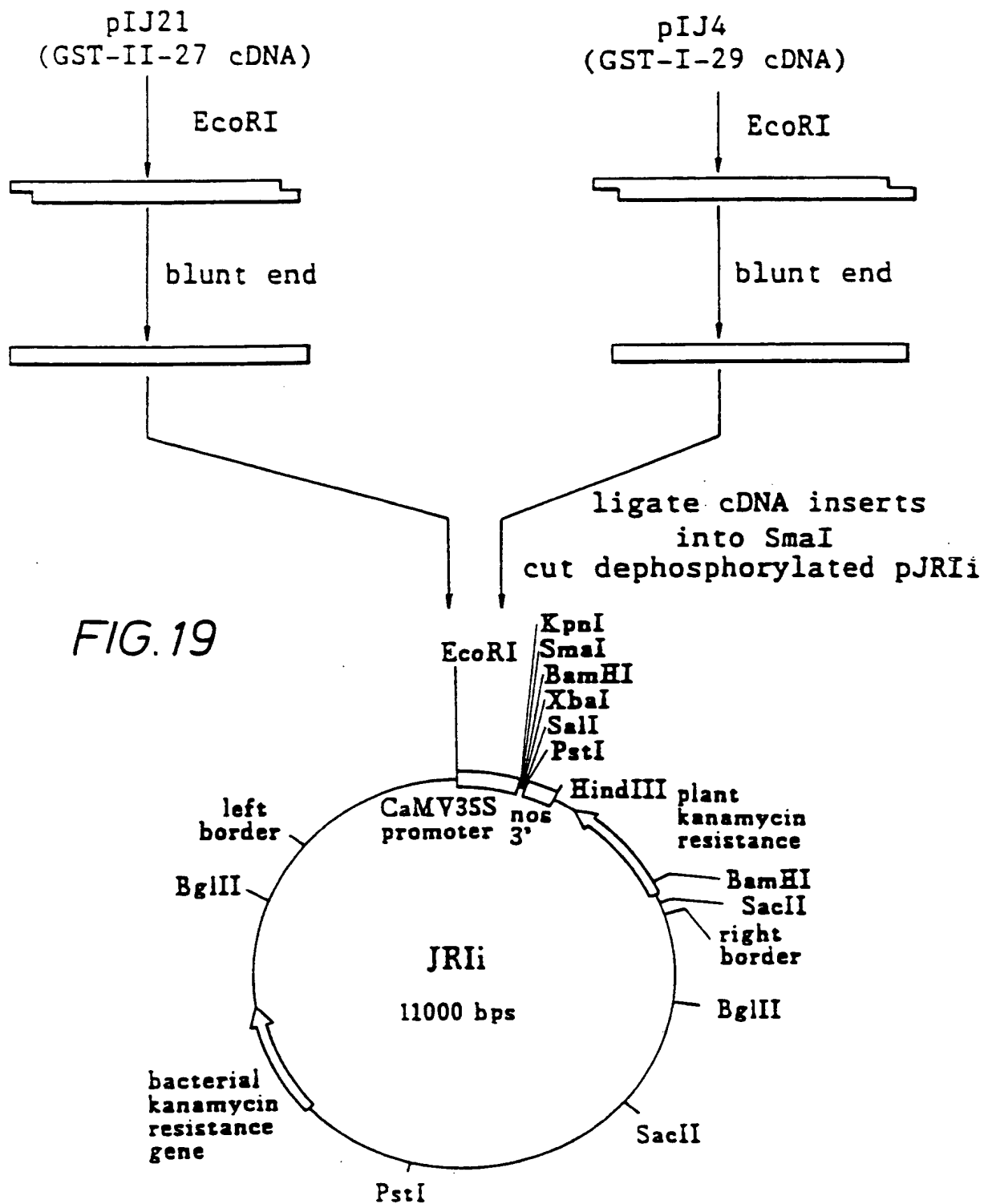
23/24

FIG. 18



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24/24



## INTERNATIONAL SEARCH REPORT

PCT/GB 92/01187

International Application No.

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/82;	C12N15/54;	C12N9/10; A01H5/00
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; A01H	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	BIOCHEMISTRY vol. 22, no. 5, 1983, pages 1068 - 1072 MOZER, T.J., ET AL. 'Purification and characterization of corn glutathione S-transferase' see the whole document	8
Y	WO,A,9 008 826 (ICI) 9 August 1990 see the whole document	1-10,13
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 28 SEPTEMBER 1992		Date of Mailing of this International Search Report 19. 10. 92
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PLANT MOLECULAR BIOLOGY. vol. 6, no. 4, 1986, DORDRECHT, THE NETHERLANDS. pages 203 - 211 SHAH, D.M., ET AL. 'Structural analysis of a maize gene coding for glutathione-S-transferase involved in herbicide detoxification' see the whole document ---	1-10
Y	WO,A,9 008 830 (ICI) 9 August 1990 see page 1 - page 7 see page 18 - page 25; figures 1,2 ---	7
X	BIOTECHNOLOGY vol. 3, July 1985, NEW YORK US pages 629 - 635 FRALEY, R. T., ET AL. 'The SEV system: a new disarmed Ti plasmid vector system for plant transformation' see page 632, right column, paragraph 1 ---	11
X	EP,A,0 256 223 (CIBA-GEIGY) 24 February 1988 see the whole document ---	11,22, 19,23,26 12,13,16
Y	GENE. vol. 76, 1989, AMSTERDAM NL pages 153 - 160 WOSNICK, M.A., ET AL. 'Total chemical synthesis and expression in Escherichia coli of a maize glutathione-transferase (GST) gene' see the whole document ---	12
Y	NUCLEIC ACIDS RESEARCH vol. 14, no. 18, 1986, ARLINGTON, VIRGINIA US pages 7227 - 7235 MOORE, R.E., ET AL. 'Cloning and expression of a cDNA encoding a maize glutathione-S-transferase gene in E. coli' see page 7227 introduction para.2 ---	16
A	PHYSIOL. PLANT. vol. 77, no. 3, 1989, pages 465 - 471 TIMMERMAN, K.P. 'Molecular characterizatio of corn glutathione S-transferase isozymes involved in herbicide detoxification' ---	1-26
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(CONTINUED FROM THE SECOND SHEET)

Form PCT/ISA/210 (extra sheet) (January 1985)

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9201187  
SA 61395

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 28/09/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9008826	09-08-90	AU-A- 4947690	24-08-90
		EP-A- 0455667	13-11-91
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		AU-A- 4945690	24-08-90
		EP-A- 0455665	13-11-91
EP-A-0256223	24-02-88	AU-A- 7314687	26-11-87
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